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**Tumour Infiltrating (TINK) and Tumour Associated
(TANK) Natural Killer cells: role in colorectal cancer
(CRC) progression and angiogenesis**

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Summary

1. SUMMARY

Substantial evidence suggests that the presence of immune cells, including Natural Killer cells (NKs), plays a crucial role in the development and/or progression of human tumors. NKs are effector lymphocytes of innate immunity, primarily involved in immunosurveillance against tumors through their cytotoxic activity. However, our previous study demonstrated that NKs are directly involved in inducing tumor angiogenesis in non small cell lung cancer (NSCLC). CD56^{bright}CD16⁻ NK cells infiltrating resectable tumors (TINKs), and even peripheral blood NK cells (TANKs) from patients with NSCLC are able to produce pro-angiogenic cytokines, including VEGF, PlGF and IL-8 showing a decidual-like phenotype.

Here, we aimed at extending our findings to colorectal carcinoma (CRC), to verify whether the TINK/TANK polarization occurs also in CRC patients and may represent a crucial feature of solid tumors.

To address our study, we performed multicolor flow cytometry, using NKs derived from peripheral blood and tissue samples of CRC patients compared with NKs derived from healthy controls' peripheral blood. Conditioned media (CM) from FACS-sorted NKs were used either for secretomic profiling, by antibody membrane array or angiogenesis functional assay on human umbilical endothelial vein cells (HUVECs).

We found that CD56^{bright}CD16⁻ NK cells predominate in CRC adjacent and tumor tissues, produce VEGF, PlGF, IL-8 and show impaired

cytotoxicity. Further, TINK/TANKs from CRC patients express the decidual NK markers CD9 and CD49a, supporting the hypothesis of a pro-angiogenic polarization. Secretomic analysis on CRC peripheral blood NKs revealed the up-regulation of several factors, including Angiogenin, Angiopoietin-1/2, TIMP-1/2, VEGF, IL-8, MMP9 and MCP-1. The activation of STAT-3 and STAT-5 pathways were observed in TANKs, suggesting a potential involvement of these signaling pathways in NK mediated induction of angiogenesis. We also demonstrated that conditioned media by FACS sorted NK cells from peripheral blood and tumor tissue of CRC patients could induce HUVEC proliferation, migration, adhesion and the formation of capillary like structures. These functional data are associated with molecular changes in HUVECs induced by NK conditioned media, that include the phosphorylation of AMP-activated protein kinase α (AMPK α), Glycogen Synthase Kinase 3 β , P70 S6 Kinase and S6 ribosomal proteins.

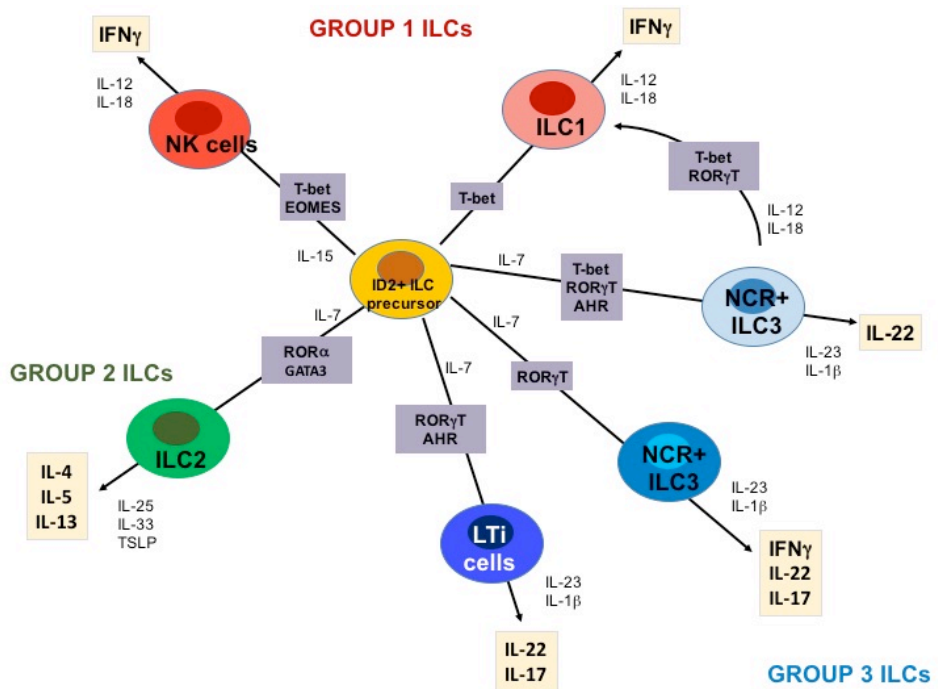
Taken together, our data demonstrate that TINK/TANKS from CRC patients are switched toward a pro-angiogenic/pro-tumor phenotype and function. We propose that TINK/TANKs could represent the hallmark for a new paradigm in CRC inflammation.

Introduction

2. INTRODUCTION

2.1 Natural Killer cells (NKs)

Natural killer (NK) cells were first discovered in 1975 by Kiessling and Herberman [1]. They belong to innate immunity, due to their ability to recognize and rapidly kill target cells without a prior sensitization [2]. Recently, NK cells have been classified as members of a specialized cohort of leukocytes, termed Innate Lymphoid Cells (ILCs) [3]. These cells have been distinguished from B and T lymphocytes based on their lack of immunoglobulin and T cell receptors, which are the result of somatic gene rearrangements [3]. Three groups of ILC have been recognized on the basis of their cytokine expression patterns and dependency on transcription factors (Figure 1): i) IFN- γ producing cells are defined as ILC1, which originally included NK cells and further distinguished from them due to the expression of CD127 (IL-7Ra) and T-bet transcription factor; ii) NK cells which depend mainly on the EOMES transcription factor and exert granule-dependent cell cytotoxicity ILC-2 which produce type 2 cytokine and need GATA-binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor- α (ROR α) for their development and functions; iii) ILC3 which depend on the ROR γ t transcription factor for their development and functions and are able to release IL-17 and/or IL-22 [3].



Adapted from Spits H et al, Nat Rev Immunol. 2013

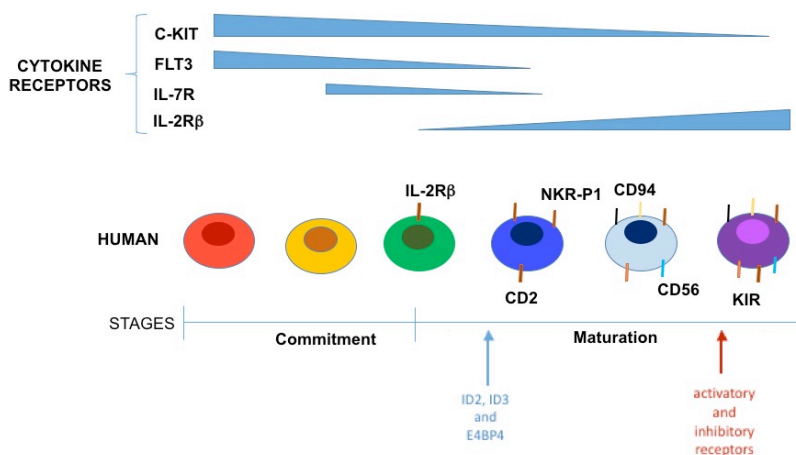
Figure 1 - ILCs family: different groups for different functions. ILC cells are conventionally divided in three groups, based on the expression of different transcription factors and cytokine release.

2.1.1 NK cell origin and development

NK cell development is characterized by numerous differentiation and maturation steps (Figure 2). NK cells originate from the CD34⁺ hematopoietic progenitor cells (HPCs) located in the bone marrow even if recent data support the idea that NK cell development is not completely restricted to bone marrow [4]. A small population of NK cells described

as CD56^{bright} NK cells has been reported to originate from hematopoietic precursor cells in the lymph nodes [5]. The developing decidua, the uterine lining during pregnancy, contains hematopoietic precursors from which decidual NK cells (dNKs) might originate [6]. NK cells have been also proposed to develop in the liver, explaining the presence of immature NK cells in adult livers [2]. A recent study suggested that NK cells could also derive from a myeloid precursor [7]. Since these findings are not completely confirmed, up to date we assume that NK cells develop from a common lymphoid progenitor (CPL) from which NK, B and T cells originate. During the first step of differentiation, the formation of a bipotent NK/T progenitor occurs, which originates NK and T cells [8]. The acquisition of the β subunit of IL-15R, also termed CD122, represents the most relevant event that characterizes the generation of NK cell precursors that acquire the ability to bind IL-2 and/or IL-15, relevant cytokines involved not only in NK cell differentiation and maturation, but also in their survival [9]. During the process of maturation from NK cell precursors to immature NK cells, several growth factor receptors, as FMS-like tyrosine kinase 3 (FLT3) and IL-7R α , are down-regulated while IL-2R β , CD2 and 2B4 are up-regulated [8]. The acquisition of NK activatory and inhibitory receptors and the activation of specific transcription factors, including ID2, ID3 and E4BP4, allow the progression from immature to mature NK cells [8]. During the last phases of maturation, NK cells are educated to recognize self-molecules.

In particular, the education process enables NK cells to distinguish between MHC class I bearing cells and those with lack of expression or altered MHC class I molecules that will be recognized and specifically killed [8].



Adapted from Colucci F et al, Nat Rev Immunol 2003

Figure 2 –NK cell development and maturation in the bone marrow. Through the acquisition of typical NK cell markers, NK cell precursors generate immature NK cells and then mature NK cells.

2.1.2 NK cell subsets

Human NK cells represent 5-15% of peripheral blood lymphocytes [4]. Human NK cells are generally defined by the expression of two surface markers, the CD3 (cluster of differentiation 3), a T-cell co-receptor that is involved in T-Cell cytotoxicity activation and CD56, which represents an adhesion molecule, in particular an isoform of the human neural cell adhesion molecule [4]. NK cells are described as CD3⁻

CD56⁺ cells.

A part from CD56 expression, human NK cells are classified based on CD16 surface antigen expression, a molecule belonging to the Ig superfamily that is a receptor for the constant portion of Ig heavy chains. CD16 is involved in the process of antibody-dependent cellular cytotoxicity (ADCC). CD16 expression correlates with different NK cell activities [4].

Peripheral NK cells are predominantly CD3⁻CD56^{dim}CD16⁺ cytotoxic NK cells, representing 90-95% of total NKs. They can release perforin, a β -pore forming toxin, and granzyme, an apoptotic cascade activator. They can also release cytokines for a brief period [10]. A minor (5-10%) counterpart of circulating NK cells is represented by CD3⁻CD56^{bright}CD16⁻ that produce high levels of some cytokines over the long term. Upon activation, these NK cells release IFN- γ and TNF α , and they kill target cells more efficiently [10]. A third distinctive NK subset has been characterized during the decidualization process, termed decidual NK cells (dNKs) [11]. This subset, which described as CD56^{superbright}CD16⁻ [12] releases large amounts of pro-angiogenic factors, including VEGF, PLGF, IL-8, and represents up to the 50% of total lymphocytes within the decidua. These dNKs play a crucial role in the modulation of maternal tolerance to an allogeneic fetus, further sustaining the angiogenic process necessary for implantation and the correct formation of spiral arteries [11] (Figure 3C). This is a clear example of “killers” that within a peculiar environment acquire “builder” functions.

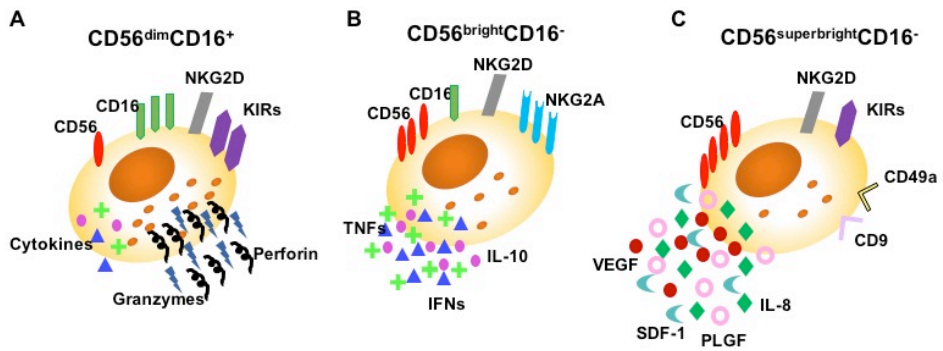
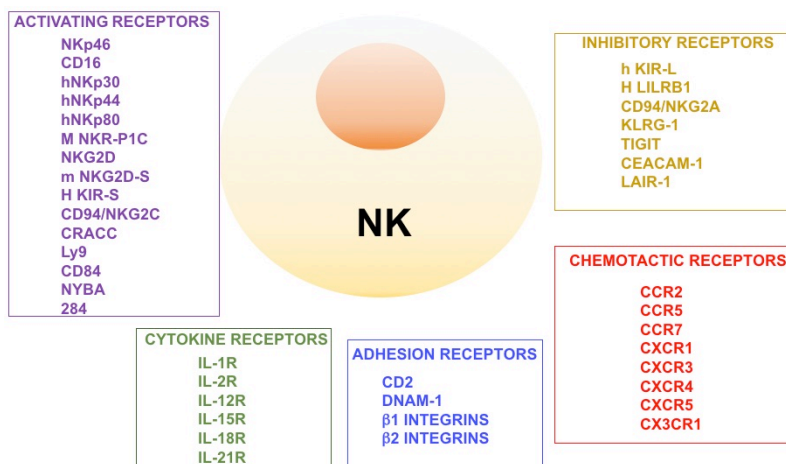


Figure 3 - Different NK cell subsets. Three different NK subset have been characterized, on the basis of distinctive surface antigen expression and described as A) CD56^{dim}CD16⁺, that represents the conventional cytotoxic NKs, characterized by a low production of cytokines and a high release of perforin and granzymes; B) CD56^{bright}CD16⁻, that are able to release a large amount of cytokines; C) CD56^{superbright}CD16⁻, that are observed in the process of decidualization and are also termed decidual NK (dNKs), which produce physiologically pro-angiogenic factors, including VEGF, IL-8 and SDF-1 and are characterized by the expression of specific surface markers as CD9 and CD49a.

NK cells express a variety of activating and inhibitory receptors (listed in Figure 4) that can modulate NK functions by stimulating or damping their responses. These molecules allow a subtle regulation of NK that lead to a balance between activating versus inhibitory signals [4]. Five classes of NK receptors have been identified and described as activating receptors, cytokine receptors and adhesion receptors, that are mainly involved in NK cell activation in terms of cytolytic activity and cytokine production. Chemotactic receptors promote NK cell recruitment to inflamed tissues, whereas inhibitory receptors mediate NK cell dampening [4].



Adapted from Vivier E. et al, Science 2011

Figure 4 - NK cell receptor repertoire. Five different classes of receptors have been identified on NK cells, including activating receptors, cytokine receptors and adhesion receptors that are mainly involved in NK cell activation towards target cells. Chemotactic receptors promote NK cell recruitment. Inhibitory receptors mediate NK cell dampening.

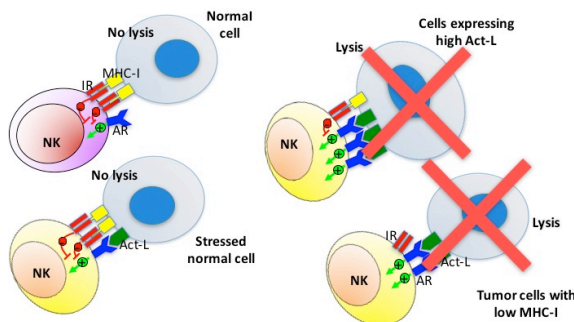
CD56^{dim} NK cells showed high levels of KIRs (Killer-cell Immunoglobulin-like Receptors) that exert mostly inhibitory effects, but low levels of CD94/NKG2 activatory receptors. On the contrary, CD56^{bright} NK cells show low to absent KIR levels and largely express CD94/NKG2A receptors [13]. Although CD56^{bright} and CD56^{dim} NK cells are comparable in terms of NKG2D expression [14] they differ in terms of NKp46 expression, which results higher in the CD56^{bright} subset [15].

2.2 NK cells and cancer progression

2.2.1 NK and tumor cells

Several *in vitro* and *in vivo* studies have shown that NK cells play a relevant role in cancer progression and patient outcome [16-19]. Cancer patients which display high numbers of tumor infiltrating NK cell have been reported to display a better prognosis [20] even if an impairment of their activities is correlated with an increased risk to develop cancer. This was demonstrated in gastric carcinoma, where a high percentage of NK cell infiltration is associated to a reduction of tumor invasion and lymph node metastasis [21]. In lung cancer, the presence of tumor-infiltrating CD11b⁺CD27⁺ NK cells positively correlated with the tumor stage and tumor size [22]. Carrega et al showed that NK cells isolated from lung cancer tissues display lower cytolytic potential as compared with NK cells from peripheral blood or normal lung tissue, even if no difference was observed in term of cytokine production including TNF- α and IFN- γ [16]. In addition, it has been reported that NK cells are particularly involved in limiting tumor metastasis pulmonary, melanoma metastasis and colorectal adenocarcinoma and peritoneal dissemination [23]. Since NK cells can recognize and kill tumor cells, several studies were performed to clarify mechanisms underlying this process. As previously described, several inhibitory receptors are expressed by NK cells able to bind MHC class I molecules. Therefore, NK cells can discriminate between self and non-self cells. During maturation phases, NK cells are

“educated”: NK inhibitory receptor repertoire is adapted to the MHC class I molecules borne by the host, assuring NK cell tolerance against self-cells. On the other hand, NK cell are simultaneously stimulated by activatory receptors, which trigger NK responses. In the presence of healthy cells, activatory signals are low, thus the binding of inhibitory receptors to MHC class I molecules is sufficient to induce NK cell tolerance (Figure 5). On the contrary, NK are able to recognize altered cells (i.e. tumor cells) that lack MHC I expression which are recognized and killed; cells expressing high levels of stress-induced ligands, even in the presence of inhibitory ligands, can be lysed if the activatory signals overcome the inhibitory ones (Figure 5) [24]. The anti-tumor activities of NK cell are not restricted to perforin and granzyme release, but include also cytokine production, and IFN- γ secretion [25].



Adapted from Bruno et al, J Natl Cancer Inst. 2014

Figure 5 - Tumor cell-mediated activation of NK cells. Three are the main mechanisms by which NK cells recognize target cells. A) Healthy cells that express MHC class I molecules induce NK cell tolerance. B) Tumor cells which lack MHC I expression are recognized and killed. C) The expression of stress-induced ligands together with the lacking of MHC class I molecules by tumor cells favor NK cell activation.

NK cell infiltrate has been observed in several types of solid malignances including melanoma [26], gastro-intestinal stromal tumors (GIST) [27], colorectal [28], renal[29], lung [30, 31], and breast cancers [32] (Figure 6). One of the major concerns regarding the role of NK cells in tumor progression is represented by the low percentage of tumor infiltrating NK cells (TINKs). Indeed, several studies in different cancer types (colorectal, lung) have shown that NK cells are mostly found around blood vessels and do not co-localize with tumor cells [31]. NKs infiltrating solid cancers have been reported to be predominately CD56^{bright} (Figure 6 and 7) [16, 30].

Tumor	NK cell infiltrate: phenotype	NK cell infiltrate: size and location
Lung adenocarcinoma	Reduced expression of Nkp30, Nkp46. Enrichment of CD56bright Perflow poorly cytotoxic NK cells	
Non-small cell lung cancer	CD56brightCD16dim infiltrating NK cells with impaired killing capability. Infiltrating CD56dim with low activating NK-rec expression and function	NKp46+ cells mainly localized at the invasive margin
Melanoma (primary)		Moderate/low CD56+CD3- cells Low CD56+NKG2D+ NK cells
Melanoma (primary/metastases)		
Melanoma (metastases)		Low CD56+ NK cells
Melanoma (nodal/skin metastases)		CD56+ NK cells rarely present in melanoma
Melanoma (nodal metastases)	NKp30, NKG2D expression inversely correlated with number of tumor cells in the LN	NK cells surround tumor cell cluster
Melanoma (nodal metastases)		Enrichment of CD56dim KIR+ CD57+ cytotoxic NK cells
Colorectal cancer		Scarce NKp46+ in itrating NK cells (despite high levels of chemokines)
Colorectal cancer	Reduced NKp46, Nkp30, DNAM-1 expression	
Colorectal cancer (lung metastases)		Low NKp46+ NK cell in ltrate
Breast cancer	Expression/function of Nkp30, NKG2D in in ltrating NK cells decreases with disease progression	
Breast cancer	Enrichment of CD56bright Perflow poorly cytotoxic NK cells	
Renal cell carcinoma (lung metastases)		High NKp46+ NK cell in ltrate correlates with improved survival
GIST (GastroIntestinal Stromal Tumors)		Substantial NKp46+ NK cell infiltrate mainly surrounding tumor nests
GIST (GastroIntestinal Stromal Tumors)		Low NK cell in ltration/high metastases at diagnosis
GIST (GastroIntestinal Stromal Tumors)		High NK cell in ltration/prolonged progression-free survival after imatinib treatment

Adapted from Cantoni et al, J Immunol Res. 2016

Figure 6 – Tumor infiltrating NKs in solid malignancies - low percentage of tumor infiltrating NK cells has been reported in several solid cancers.

In the last decade, several studies have demonstrated that cancer cells are able to decrease NK recruitment and impair NK cell anti-tumor activities, promoting the “tumor escape phenomenon” [33]. By inhibiting NK cell recruitment, tumor cells prevent NK cell cytotoxic functions. In addition, tumor cells could also down-regulate the expression of NK activatory receptors, promoting their desensitization, tumor immunotolerance and promote the induction and the release of immunosuppressive microenvironment by producing TGF- β and IL-10, finally dampening NK cell response [34]. The ability of tumor cells to attenuate NK anti-tumor activity and enhance pro-tumor properties is a crucial phenomenon, termed “polarization” (Figure 7). Given their plasticity, diverse cell of the immune system, both of innate and adaptive immunity, have been described to acquire pro-tumor/pro-angiogenic phenotype and functions. Macrophages represent the most characterized immune cell component in this scenario. Studies on NK cell polarization are still few in the literature and the major of them are focused on NK anergy and impaired toxicity [16-19, 22]. The first study in investigating the contribution of NK cells to tumor progression by sustaining angiogenesis was done in non small cell lung cancer (NSCLC) [30, 33]. The NK cells infiltrating tumors were termed tumor infiltrating NK cells (TINKs), while peripheral blood NK cells were termed tumor associated NK cells (TANKs) [30, 33].

2.2.2 NK and angiogenesis

Angiogenesis, i.e. the formation of new blood vessels, represents a necessary process in a wide number of physiological (reproduction, development, tissue repair and wound healing) events and pathological conditions, including neurodegenerative diseases, diabetes and cancer [35]. NK cell contribution to angiogenesis has been reported in different contexts [33]. As previously described, a peculiar CD56^{superbright}CD16⁻ NK subset found in the decidua during implantation acquires low cytotoxic and high pro-angiogenic activities [36, 37]. NK cells have also been reported to correlate with angiogenesis during tissue repair process in c-kit deficient mice [38]. Further analyses showed that lectin-like receptor 1⁺ and $\alpha 4\beta 7$ integrin⁺ NK cells were required for angiogenesis in the damaged murine myocardium [39]. In models of bFGF (basic-fibroblast growth factor)-induced corneal and laser-induced choroidal angiogenesis, a reduction of NK infiltrate, led to a significant decrease of neovascularization[40].

To date, studies investigating the direct contribution of NK cells in promoting angiogenesis have been mostly addressed on the cardiovascular implications of myocardial NK cells, or on ocular NK properties and on dNK cells, as reported in Figure 7 [30]. Regarding the role of NK cells in sustaining tumor angiogenesis, very few studies have focused their attention on the link between tumor NK infiltrate and vessel density [33]. Previous findings by our lab in the non small cell lung cancer (NSCLC) context have shown that the major NK subset in NSCLC

tissues is the decidual-like CD56^{bright}CD16⁻ [30]. When examined for angiogenic cytokine production it was showed that TANKs from squamous cell carcinoma patients produced substantially higher levels of VEGF and PlGF as compared with those of adenocarcinomas that resulted comparable with those from healthy controls [30]. NSCLC TINKs showed angiogenesis-associated activities *in vitro*, inducing the formation of capillary-like structure by endothelial cells and promoting their migration [30].

NK CELL SYSTEM	MODELS	SUMMARY
dNK cells	Human endometrium	Human endometrium expresses a wide range of angiogenic growth factors (VEGF-A, PlGF, Ang1 and Ang2). Intense hybridization for VEGF-C, Ang2 and PlGF mRNAs was found in uterine NKs during the secretory phase endometrium. IL-2 and IL-15 up-regulated VEGF-C, but not PlGF or Ang2 mRNA levels in isolated NK cells. Conditioned medium from decidual NK cells did not induce human umbilical vein endothelial cell apoptosis.
	murine uterus	NKs have been demonstrated to be involved in angiogenesis in the pregnant uterus and contribute to the pregnancy maintenance by increasing VEGF production.
	Human and murine decidual NK cells	dNK cells are able to regulate trophoblast invasion both in vitro and in vivo by promoting IL-8 and interferon-inducible protein-10 chemokines production. dNK cells have also been shown to release a wide array of angiogenic factors and induce vascular growth in the decidua.
Myocardial NK cells	c-kit deficient mice	c-kit-deficient mice showed dysfunctional NKs and impaired myocardial repair ability and angiogenesis
	SCID-NOD/SCID mice	SCID-NOD/SCID mice showed dysfunctional NKs and impaired myocardial repair ability
Ocular NK cells	asialo-antibody NK depletion in mice	IFN- γ -secreting NK cells are able to induce angiogenesis by promoting enhanced VEGF expression by macrophages
Tumor Infiltrating/Tumor associated NKs	Human NSCLC	The CD56 bright CD16(-) NK cell subset displayed activation markers including Nkp44, CD69, and HLA-DR and showed an impaired cytolytic potential.
	Human NSCLC	Intratumoral NK cells displayed an altered phenotype that was associated with relevant defects in the ability to activate degranulation and IFN- γ production.
	Human breast cancer	Tumor infiltrating and tumor associated NKs showed a downregulation of KIR and impaired cell function and cytotoxicity. TGF β 1 and PGE2 were associated with decreased NK function and tumor progression.
	Human colon cancer	Tumor infiltrating NKs cells showed reduced IFN γ production and down- regulation of CD161, CD94, CD158b, Nkp30, NKG2D, DNAM-1, and CD16. The altered phenotype was associated with decreased cytotoxic abilities.
	Human NSCLC	NK cells from NSCLC patients produce angiogenic factors including VEGF and PlGF and are able to induce endothelial cell recruitment and morphogenesis ex vivo.

Adapted from Bruno et al, J Natl Cancer Inst. 2014

Figure 7 - Studies that have focused on the pro-angiogenic and pro-tumor role of Natural Killer cells. Several studies have investigated the role of NK in tumor progression and in angiogenesis. Most them, addressed their attention on decidual NK or the cardiovascular compartment. Only few studies have evaluated the role of NK in tumor associated angiogenesis.

Recently, Gotthard et al. highlighted the relationship between NKs and angiogenesis, demonstrating that *Stat5^{Δ/Δ}Ncr1-iCre^{Tg}-Vav-Bcl2* mice failed to control tumor growth of RMA-S lymphoma cells and a *v-abl* transformed tumor clarifying the significant tumor-promoting function of STAT5-deficient NK cells [41]. Analyzing the levels of proangiogenic factors in this murine model, they observed an increased VEGF production by STAT5-deficient NK cells. To elucidate the role of VEGF production in NKp46⁺ cells, Gotthardt and colleagues established *Vegfa^{Δ/Δ}Ncr1-iCre^{Tg}* mice, characterized by NKp46⁺VEGF⁻ cells. By using different tumor models (*v-abl*⁺ tumor, RMA-S, and A-MuLV-induced leukemia), they showed a significant reduction of tumor burden and fewer CD31⁺ blood vessels in tumors [41, 42].

2.2.3 Clinical and preclinical anti-cancer therapies able to modulate NK activity

Several targets of current anti-cancer therapies are expressed by tumor cells as well as immune cells. Therefore, few treatments not only impact on cancer cell survival and proliferation but also influence the immune system activity [43]. It has been demonstrated that radiotherapy or chemotherapy, including arabinofuranosyl cytidine (Ara-C), cisplatin, or 5-fluorouracil (5-FU), can increase the expression of some NK cell activating ligands enhancing NK cell recognition and killing [44].

More recently, several target therapies have been demonstrated to improve NK cell-mediated tumor killing [45, 46]. The proteasome inhibitor bortezomib induced the expression of ligands of NK cell activating receptors. Lenalidomide, an anti-angiogenic drug, modulates the immune response indirectly by enhancing tumor cell ligands and inducing the expression of NK cell stimulatory cytokines (i.e. T cell derived IL-2) or directly by lowering the threshold for NK cell activation [47, 48].

During the last years, several studies have also focused their attention to checkpoint inhibitors as one of the most promising approach among immunotherapies. It has been demonstrated that treatments with anti-CTLA4 or anti-PD-1 antibodies can restore T cell activity in cancer patients resulting in tumor regression in some patients [43]. Benson et al also showed that NK cells from multiple myeloma and renal carcinoma patients expressed PD-1 on their surface and CT-011, a novel anti-PD-1 antibody, is able to enhance human NK-cell function against autologous, primary MM cells [49].

2.3 Colorectal Cancer

2.3.1 Epidemiology and pathologic features of Colorectal Cancer (CRC)

Colorectal cancer (CRC) represents the third most common cancer in men (746,000 cases, 10.0% of all cancers in men worldwide) and the

second most common cancer in women (614,000 cases, 9.2% of all cancers in women worldwide) [50]. Regarding incidence and mortality rates, there is a marked variation across countries and regions [51]. Eastern Europe and Asia have observed an enhancement of incidence rates that have been attributed to behavioral risk factors related to diet and lifestyle. Some countries, including Brazil, Mexico, and Romania have observed increasing CRC mortality rates that might be correlated with the limited healthcare resources [51].

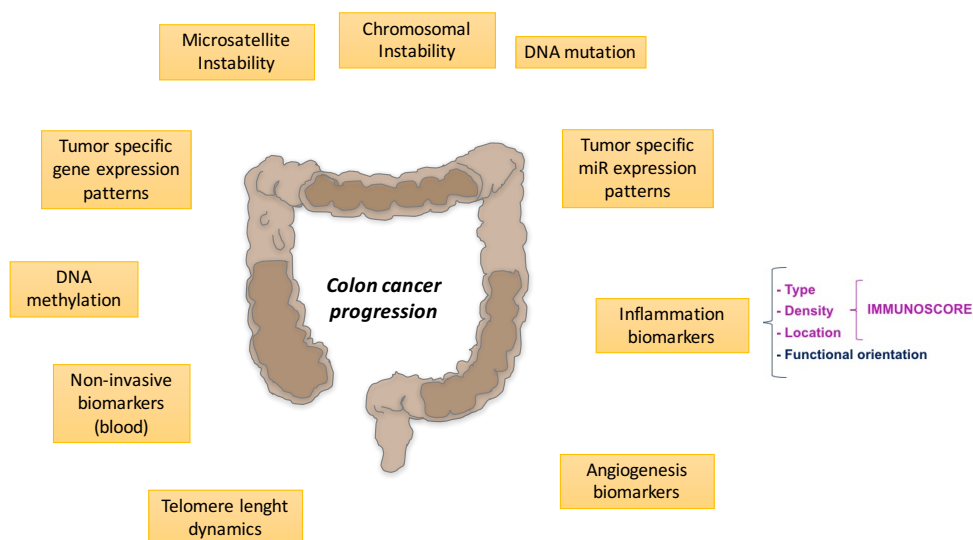
The World Health Organization (WHO) distinguished a wide number of CRC histological variants that includes mucinous adenocarcinomas, signet ring cell adenocarcinomas, medullary, micropapillary, serrated, cribriform comedo-type, adenosquamous, spindle cell, and undifferentiated [52]. Mucinous adenocarcinomas are defined by >50% of the tumor volume composed of extracellular mucin and are characterized by large glandular structures with pools of extracellular mucin. The most of mucinous adenocarcinomas occurs in patients with hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) with high-levels of microsatellite instability (MSI-H), while mucinous adenocarcinomas that present microsatellite stability (MSS) are reported to be more aggressive [53]. Signet ring cell adenocarcinomas are rare in the colorectum, representing only 1% of all colorectal carcinomas and are defined by the presence of >50% of tumor cells showing signet ring cell features characterized by a prominent intracytoplasmic mucin vacuole.

Conventionally, signet ring cell carcinoma are poorly differentiated (high grade) even if some of them can be MSI-H tumors displaying a lower grade [54]. Medullary carcinomas are extremely rare (5-8 cases for every 10,000 colorectal cancers) characterized by sheets of epithelioid neoplastic cells with large vesicular nuclei, prominent nucleoli, and abundant cytoplasm and is strongly associated with MSI-H. It usually has a favorable prognosis despite its poorly differentiated or undifferentiated histology [55].

Randomized controlled trials (RCT) have shown that screening is associated with a reduction in CRC mortality. Indeed, CRC incidence and mortality rates have been declining in the United States and other high-income countries due to the introduction of screening programs that enables primary prevention and early detection [50, 51]. During the last decade, technological advances have led to the development of new, less invasive screening approaches, including fecal immunochemical testing, computed tomographic colonography (CTC), stool DNA testing, and colon capsule endoscopy [51]. In addition, several studies focused their attention in order to identify possible non-invasive biomarkers (blood- or stool-based markers) for early detection of CRC able to ameliorate CRC diagnosis, improve patient prognosis, prediction of treatment response, and possible prediction of recurrence risk [56].

The immune contexture (including type, density, and location of immune cells within the tumor samples) of tumor microenvironment has been

shown to influence tumor progression determining the fate and survival of the patient and the response to therapy. Thus, several studies have focused on novel and non-invasive prognostic tools able to measure in situ immune cell infiltrates. The immunoscore, a scoring system based on the quantitated numbers of cytotoxic and memory T cells infiltrating the core of the tumor and its invasive margins, revealed the major positive role of these cells for patient's survival, suggesting its potential application as a biomarker for the prediction of prognosis and response to therapy [57] (Figure 8). In CRC context, an Immunoscore based on the combined analysis of CD8⁺ plus CD45RO⁺ cells in specific tumor regions has been shown as a relevant tool to predict tumor recurrence and survival in patients with early-stage (AJCC/UICC-TNM stages I and II) CRC. Moreover, considering the disease-specific survival according to CD8⁺ and CD45RO⁺ densities in combined tumor regions (central tumor/invasive margin, CT/IM), the scoring system allows the discrimination between patients' subgroups characterized by distinct clinical outcomes in terms of disease-free survival and overall survival [58]. The relevance of the localized immune reaction in predicting recurrence and survival in patients with early-stage colorectal carcinoma has also been assessed [59].



Adapted from Aghagolzadeh P et al, World J Gastroenterol. 2016

Figure 8 - CRC Biomarkers – Several studies have focused on the identification of novel and non-invasive biomarker discovery for CRC based on the personalized genotype and clinical information that might facilitate the classification of patients to improve early detection and ameliorate patients' outcome.

2.3.2. Colorectal cancer and NKs

It is now clear that chronic inflammation represents an hallmark of cancer [60] and a key risk factor for CRC development [61-63]. The use of anti-inflammatory agents has been associated with a decreased CRC incidence [64]. On the contrary, some studies have demonstrated that an increased percentage of immune cell infiltration represents a good

prognostic factor for clinical outcome [57, 65], suggesting a controversial role of immune cell infiltration in CRC.

However, given the strong correlation between colorectal cancer and inflammation, several studies during the last decades have deepened the contribution of the diverse immune cell types in the CRC progression [66, 67]. Tumor associated macrophages (TAMs) contribution to colon cancer depends on their polarization state: peri-tumor M1 macrophages [68] (TAM1) are able to prevent tumor development whereas M2 polarization [69] (TAM2) appears to be associated with cancer promotion by increasing of pro-tumor factors. TAM2 released IL-1 β increases HIF production and activates NF- κ B-dependent PDK1/AKT pathway in tumor cells, inactivates GSK3 β and enhances Wnt signaling leading to colon cancer cell growth [70, 71].

NK cells have been found as a minor component in the overall CRC inflammatory infiltrate the CRC tissues [28, 62]. Halama et. al have investigated NK percentage in CRC tissues, performing a quantification of infiltrating NK and T cells as far as the chemokine/cytokine related production in colon cancer biopsies, adjacent-normal and tumor tissues and liver metastases. The data obtained with this study clearly indicated that despite high levels of chemokines, the number of NK cells was limited within CRC biopsies, suggesting that cytokine production was mainly due to T cell infiltrate. On the contrary, NK percentage in adjacent normal tissues was significantly higher than in CRC tissues [72].

However, other studies demonstrated that, despite their low number in tumor tissues, CRC infiltrating NKs can influence patient outcome [62, 73]. Sconocchia et al demonstrated a significant cross talk between intra-tumor NK cell infiltration and T lymphocytes within CRC, showing that NKs positively influenced the clinical outcome of CRC patients by enhancing the protective role of tumor-infiltrating CD8⁺ T cell [62].

Rocca et al characterized tumor infiltrating NKs in CRC tissues and peripheral blood (PB), demonstrating a relevant alteration of their phenotype, with a significant down-regulation of several receptors, including CD16, NKG2D, NKp30, NKp44, NKp46, CD161 and DNAM-1 [74]. They also observed an impairment of CRC and PB NK functions, including their ability to activate degranulation and IFN- γ production [74]. Performing co-culture experiments, they also demonstrated that phenotypic modulation and functional impairment of NK cytotoxic properties was promoted by cancer cells, supporting the hypothesis of the pro-tumor polarization induced by cancer cells on NKs [74].

Aim of the study

3. AIM OF THE STUDY

Substantial evidence suggests that the presence of immune cells plays a crucial role in the development and/or progression of human tumors. CRC represents an extremely heterogenic “*scenario*” where several “*actors*” interact with each other. In the tumor microenvironment (TME) context, immune cells can be considered a “double edged sword” that can either destroy tumor cells or promote tumor growth and dissemination according to their polarization state. Recently we have identified a new NK subset in non small cell lung cancer patients (NSCLC), characterized by a decidual-like CD56^{bright}CD16⁻VEGF^{high}PIGF^{high}IL-8⁺IFN γ ^{low} phenotype, that we termed tumor infiltrating (TINKs) and tumor associated NKs (TANKs). Based on these results, we are now interested in extending our findings in colorectal carcinoma to:

1. Evaluate whether the same TINK/TANK polarization also occurs in CRC patients.
2. To confirm the presence of a predominant CD56^{bright}CD16⁻NKG2D⁻ phenotype in CRC NK cells that has been already described in literature.
3. Analyze cytokines production by NK cells isolated from CRC patients and clarify their contribution in tumor angiogenesis induction.

4. Evaluate the possible to employ TINK/TANKs as CRC biomarkers.

Material and Methods

4. MATERIALS AND METHODS

4.1 Samples selection and patients' characteristics

Samples, including peripheral blood (PB), tumor tissues (TUM) and normal adjacent tissues (ADJ) were obtained from CRC patients upon informed consent in an institutional ethics committee approved study in collaboration with the *Ospedale di Circolo di Varese*. Tissue samples, after surgical resection, were maintained in PBS 1X (Sigma-Aldrich, St Louis, MO) with 1% penicillin/streptomycin (Sigma-Aldrich) while 15-20 ml of PB samples were drawn from the same patients before surgical intervention into heparinized blood collection tubes. All samples were stored at 4°C before processing. Patients with diabetes, human immunodeficiency virus (HIV)/hepatitis C virus (HCV)/hepatitis B virus (HBV) infection and chronic inflammatory conditions are excluded. Patients previously treated with chemotherapy or radiotherapy, or those iatrogenically immunosuppressed or having undergone myeloablative therapies were also excluded from the study. Our cohort included 6 patients with non-oncologic bowel disease (diverticular diseases – Table 1) and 57 oncologic patients with colorectal cancer, whose characteristics are summarized in Table 2. Peripheral blood samples from age-matched healthy donors (HC) were used as controls.

A)

ID	GENDER	AGE	SMOKING
1°	F	35	NO
2°	M	33	NO
3°	M	30	NO
4°	F	84	NO
5°	F	31	YES
6°	F	29	NO
7°	M	29	YES
8°	M	58	NO
9°	M	67	NO
10°	M	46	NO

B)

ID	GENDER	AGE	HYSTOLOGY
I	M	73	diverticular disease
II	M	42	diverticular disease
III	F	87	diverticular disease
IV	M	60	diverticular disease
V	F	68	diverticular disease
VI	M	62	diverticular disease
VII	F	66	diverticular disease

Table 1 – Clinical characteristics of A) healthy controls and B) non oncologic patients.

ID	GENDER	AGE	HYSTOLOGY	TNM	STAGE	SMOKING	FAP/HNPCC	MSI	MUTATIONS	TOPOGRAPHY
1	M	77	moderately differentiated adk	pT3pN2	G2	NO	/	/	/	rectum
2	F	67	moderately differentiated adk	pT3pN0	G2	NO	/	/	/	sigmoid
3	F	77	moderately differentiated adk	pT3pN0	G2	NO	/	/	/	rectosigmoid junction
4	F	83	mucinous adk	pT3pN0	G2	FORMER	/	hMLH1 e hPMS2 N.O.	MSI in all loci	colon dx
5	F	84	adk	T3N0	G2	ND	ND	ND	ND	sigmoid
6	M	71	moderately differentiated adk	pT3pN1	G2	FORMER	/	/	/	rectosigmoid junction
7	M	77	moderately differentiated adk	pT3N2	G3	NO	/	/	/	rectosigmoid junction
8	M	80	moderately differentiated adk	pT3pN0	G2	FORMER	/	/	/	rectum
9	F	63	moderately differentiated adk	pT3pN0	G2	YES	/	/	/	rectosigmoid junction
10	M	86	moderately differentiated adk	pT3 pN1 (1/48)	G2	YES	/	/	/	hepatic flexure
11	F	57	moderately differentiated adk	pT3pN0	G2	NO	/	/	/	sigmoid
12	M	86	tubulovillous adenome	pT3pN0	G2	FORMER	/	/	/	colon dx
13	F	75	ADK	pT2pN0 pM1	G2	NO	/	/	KRAS not mut	rectosigmoid junction
14	M	100	adk	pT1Nx	G2	NO	/	/	/	rectum
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	F	85	ND	?	?	ND	ND	ND	ND	ND
18	F	76	moderately differentiated adk	pT3pN0 pM0	G2	YES	/	/	/	sigmoid
19	M	79	moderately differentiated adk	pT3pN1	G2	FORMER	/	/	/	colon dx
20	M	66	ND	?	?	ND	ND	ND	ND	ND
21	F	63	mucinous adk	pT3pN0	G2	FORMER	/	hPMS2 N.O.	/	destro
22	M	60	poorly differentiated adk	pT3m pN2b	G3	NO	/	/	/	transverse colon
23	M	80	moderately differentiated adk	pT3pN0	G2	YES	/	/	/	colon dx
24	M	77	moderately differentiated adk	ypT3yp N2a	G2	NO	/	/	/	rectum
25	M	60	moderately differentiated adk	pT2 pN0	G2	FORMER	/	/	/	rectum
26	F	69	moderately differentiated adk	pT3pN0	G2	FORMER	/	/	/	colon dx
27	M	48	moderately differentiated adk	pT3pN0	G2	NO	Lynch Syndrome	hMSH6 (Clone 44) N.O. present MSI in all loci	/	colon sx
28	M	83	tubulovillous adenome		/	NO	/	/	/	colon dx and sigmoid
29	M	62	moderately differentiated adk	pT3pN1 a	G2	FORMER	/	/	/	transverse colon

Table 2A – Clinical characteristics of CRC patients included in our study

ID	GENDER	AGE	HISTOLOGY	TNM	STAGE	SMOKING	FAP/HNPCC	MSI	MUTATIONS	TOPOGRAPHY
30	M	84	moderately differentiated adk	pT3pN0	G2	YES	/	/	/	colon dx
31	M	76	moderately differentiated adk	pT3pN0	G2	FORMER	/	/	/	splenic flexure
32	F	59	mucinous adk	pT3pN0	G2	NO	/	no	/	hepatic flexure
33	F	75	adk	pT4bpN 2aM1	G2	NO	/	/	K-RAS mut	rectosigmoid junction
34	M	53	mucinous adk	pT3 pN0	G2	NO	/	N.O. MSI in all loci	N-RAS e K-RAS not mut	sigmoid
35	M	75	moderately differentiated adk	pT3pN0	G2	YES	/	/	/	colon dx
36	M	62	moderately differentiated adk	pT3pN0	G2	NO	/	/	/	sigmoid
37	M	68	mucinous adk	pT3pN1 bM1	G2	NO	/	/	mut KRAS and not mut NRAS	sigmoid
38	F	86	?	?	?	?	/	/	/	/
39	F	68	tubulovillous adenome	pT 1	pN 2b	/	/	/	/	/
40	F	74	undifferentiate adk	pT 3	pN 0	/	/	hMSH2 (Clone G219-1129): absent hMSH6 (Clone 44): absent hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	KRAS mutated (pG12V (c.35G>T))	/
41	M	76	moderately differentiated adk	pT 4a	pN 0	/	/	hMSH2 (Clone G219-1129): Present, reduced hMSH6 (Clone 44): Present, reduced hMLH1 (Clone M1): Present, reduced hPMS2 (Clone EPR3947): Absent	/	/
42	M	79	tubulovillous adenome			/	/	/	/	/
43	M	70	mucinous adk	pT 2	pN 0	/	/	/	/	/
44	F	68	moderately differentiated adk	pT 2	pN 0	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	/
45	F	82	moderately differentiated adk	pT 3	pN 1b	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	rectum

Table 2B – Clinical characteristics of CRC patients included in our study

ID	GENDER	AGE	HYSTOLOGY	TNM	STAGE	SMOKING	FAP/HNPCC	MSI	MUTATIONS	TOPOGRAPHY
46	F	80	mucinous adk	pT 3	pN 1a	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Absent hPMS2 (Clone EPR3947): Absent	/	transverse colon
47	M	92				/	/		/	colon dx
48	F	42	mucinous adk	pT 3	pN 0	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	
49	M		moderately differentiated adk			/	/		/	rectum
50	M	73	mucinous adk	pT 3	pN 0	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	rectum
51	F	77	moderately differentiated adk	pT 2	pN X	/	Lynch Syndrome	hMSH2 (Clone G219-1129): Absent hMSH6 (Clone 44): Absent hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	transverse colon
52	F	76	moderately differentiated adk	pT 3	pN 2b	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	cecum
53	F	82	adk			/	/		/	cecum
54	F	79	poorly differentiated adk	pT 3	pN 1c	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Absent hPMS2 (Clone EPR3947): Absent	/	ascending colon
55	M	67	moderately differentiated adk	pT 4a	pN 1a	/	/	hMSH2 (Clone G219-1129/FE11): present hMSH6 (Clone 44): present hMLH1 (Clone M1/G168 15): present hPMS2 (Clone EPR3947 / A16-4): present	/	/
56	M	72	mucinous adk	pT 3	pN 0(+)	/	/	hMSH2 (Clone G219-1129): Present hMSH6 (Clone 44): Present hMLH1 (Clone M1): Present hPMS2 (Clone EPR3947): Present	/	rectum
57	M	62	moderately differentiated adk	pT 3	pN 1a	/	/	h-MLH1: present h-MSH2: present h-PMS2: present h-MSH6: present	/	rectosigmoid junction

Table 2C – Clinical characteristics of CRC patients included in our study

4.2 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were obtained by diluting whole blood samples, either from CRC patients, patients with diverticulosis or healthy controls, 1:2 with PBS 1X and subsequently subjected to a density gradient stratification. Briefly, diluted whole blood samples was carefully layered onto Histopaque-1077 Ficoll (Sigma-Aldrich) and centrifuged at 500 x g for 30 minutes at room temperature without brake. Finally, the lymphocyte-enriched ring at the interface was transferred into a new collection tube and washed with PBS 1X by centrifugation at 250 x g for 5 minutes.

4.3 Solid Tissue Enzymatic Digestion

Biopsies (TUM and ADJ) were extensively washed in PBS 1X to remove residual red blood cells, debris and dead cells. Necrotic tissue and areas occupied by adipose tissue were removed using scissors. Tissues were then mechanically dissociated with scissors to obtain small fragments further subjected to enzymatic digestions for 1 hour at 37°C, using a cocktail containing Collagenase type II (2 mg/mL, Sigma Aldrich). The total cell suspension obtained was then filtered through 40 µm cell strainers (Becton Dickinson (BD) San Jose, CA), then washed twice by centrifugation in RPMI 1640 (EuroClone, Milan, MI) with 10% FBS (Sigma Aldrich), 1% glutamine Gln, 1% Pen/Strep to remove residual enzymes.

4.4 TINK/TANK isolation and culture

Natural Killer (NK) cells were isolated from PBMCs obtained from PB and biopsies as previously described by using a 3 laser FACS-Aria II BD sorting device. Briefly, cell suspension was incubated with a cocktail of antibodies that included: FITC-CD45/APC-CD14, PerCP-CD3, APC-CD56 (all purchased by Miltenyi Biotec, Auburn, CA) and PE-ROR γ t (R&D System, Minneapolis, MN) for ADJ and TUM derived cells (Figure 9). For secretomic analyses, immunoblotting and functional assays, the purity of the NK isolated cells was always verified by FACS.

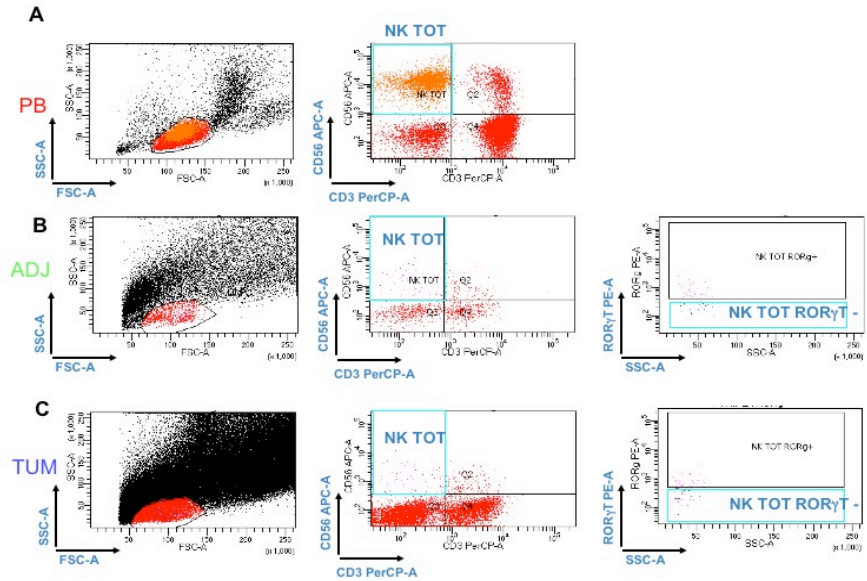


Figure 9 – Gating strategy for NK selection from peripheral blood (PB), adjacent normal tissue (ADJ) and tumor tissue (TUM). Following FSC/SSC physical parameter setting, total NKs were identified as CD45⁺CD14⁻CD3-CD56⁺ cells (A-C). For tissue samples (B,C), a further step for NK identification was performed by RoRyt⁺ cell exclusion from CD45⁺CD14⁻CD3-CD56⁺ cells.

4.5 Phenotype and Functional Characterization of TINK/TANK from CRC patients

To assess NK cell subset distribution, 2.5×10^5 PBMCs from PB (HC and patients), TUM and ADJ tissues were stained for 30 minutes at 4°C with monoclonal antibodies (mAbs) in a direct immunofluorescence assay and assessed by flow cytometry (FACS Canto I; BD), as follows: FITC-CD45/APC-CD14, PerCP-CD3, APC-CD56, FITC-CD16, PE-CD9, PE-CD49a, FITC-NKG2D (Miltenyi Biotec). FITC-conjugated, PE-conjugated, PerCP-conjugated, and APC-conjugated isotype mAbs were used as controls. After physical parameter setting (Forward and Side Scatter) total lymphocytes were identified as CD45⁺CD14⁻ cells and gated for further assessment for CD3⁻ and CD56⁺ cells. CD16 expression was evaluated on CD3⁻CD56⁺ (total NKs) gated cells. Finally, the expression of the decidual markers CD9 and CD49a, and the NKG2D activatory marker was evaluated both on total and CD56^{bright}CD16⁻ NKs. For intracellular cytokine detection, 1×10^6 PBMCs from PB (HC and patients), TUM and ADJ tissues were cultured overnight in RPMI 1640 supplemented with 10% heat-inactivated FBS (EuroClone), Pen/Strep, and IL-2 (100 U/ml; R&D Systems, Minneapolis, MN) at 37°C and 5% CO₂. Cells were stained for NK cell surface markers, as already described, washed with PBS 1X and treated with Cytofix/Cytoperm fixation and permeabilization kit (BD) for 10 minutes at 4°C. Cells were then washed in PBS and stained with directly conjugated antibodies

(listed in Table 4 A) for 30 minutes. For indirect staining, cells were incubated for 1 hour at 4°C with primary unlabeled antibodies (listed in Table 4 B), washed and then stained with secondary labeled antibody (4°C, 30 minutes, dark). The fluorescence of each antibody was set on its appropriate isotype control and for indirect staining the secondary antibody alone was used as a negative control.

A) Direct staining

Antigen	Clone	Fluorochrome	Company
VEGF	23410	PE	R&D
IL-8	6217	PE	R&D
MCP-1	2H5	PE	Biolegend

B) Indirect staining

Primary Antibody	Primary Antibody Specie	Company	Secondary Antibody	Secondary Antibody Fluorochrome	Company
ANGIOGENIN	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
ANGIOPOIETIN 1	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
TIMP-1	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
TIMP-2	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
MMP-9	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
STAT-3	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
Phospho STAT-3	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
STAT-5	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D
Phospho STAT-5	Rabbit anti-human	Abcam	Goat anti-rabbit	PE	R&D

Table 4 – Antibody for Intracellular staining (Flow Cytometry) – A) anti-human directly conjugated antibodies used for FACS analysis; B) Primary and secondary antibodies exploited for indirect FACS staining.

4.6 Secretomic analysis of tumor-associated NKs

Secretomic studies used 99% pure FACS-sorted NKs (CD3⁻ CD56⁺), isolated from total PBMCs from healthy controls and CRC PB, obtained as above.

CRC-TANKs and NK cells from controls were transferred for 24 hours in FBS free RPMI 1640 to obtain conditioned media. After 24 hours, supernatants were collected, centrifuged to deplete residual dead cells and debris and concentrated using the Centricon devices (Millipore, Temecula, CA) with a 3kDa membrane pore cut-off (conditioned media; CM). The ability of CRC TANKs to release pro-angiogenic factors was assessed by secretomic analysis, using the Human Angiogenesis Array C1000 (RayBiotech, Inc., Norcross GA). A full list for the spotted detection antibodies is available at web site www.abcam.com. Briefly, following membrane blocking for 30 minutes using the buffer provided by the kit, an equal amount of HC and TANK CM concentrated was diluted up to 1 mL with blocking buffer 1X and incubated overnight with membranes. Membranes were then washed to remove unbound material and incubated with a biotinylated antibody cocktail for 2h at room temperature. Membranes were washed and incubated with the HRP-streptavidin concentrate for 2 hours. Following a washing step, chemiluminescent detection reagents were added. Luminescent signals were captured by exposure to Amersham Hyperfilm. Arrays were scanned into a computer and optical density measurements were obtained with National Institutes of Health ImageJ software.

4.7 NK degranulation assay

CD107a expression on NK cells was measured to assess NK cell degranulation. PBMCs, isolated from peripheral blood, either from CRC patients or healthy controls, as well as from CRC adjacent normal and tumor tissues, were incubated with or without K562 cells in 200 μ L at 37°C, 5% CO₂, in 1:5 effector/target ratio. K562 cells are a human erythroid leukemia cell line expressing low levels of MHC class 1 widely used for the cytotoxicity assay. Following 4-hour culture, cell mixture was stained with mAbs against CD45-FITC, CD14-PE CD3-PerCP, CD56-APC. Total lymphocytes were gated as CD45⁺CD14⁻ cells, and NK cells were further counted as CD45⁺CD3⁻CD56⁺ cells. To determine the CD107a expression of NK cells, CD107a positive rate of CD3⁻CD56⁺ NK cells was analyzed. Killing efficiency was normalized to resting NK cells (peripheral blood, adjacent and tumor tissues, without the presence of K562 cells) for patients as compared to healthy controls.

4.8 Protein extraction and pathscan sandwich immunoassay

The PathScan Intracellular Signaling array kit (Cell Signaling Technology, Danvers, MA) was used, according to the manufacturer's instructions, to detect the activation of intracellular signaling pathways. Briefly, NKs isolated as previously described from healthy donors and CRC patients were lysed in 1X Cell Lysis buffer. Following membrane blocking for 15 minutes using the buffer provided by the kit, 25 μ g of

total protein lysates were added to each well and incubated for 2 hours at RT. Membranes were then washed for 5 times and then incubated with the detection antibody cocktail provided by the kit for 1 hour at RT. Following a washing step, HRP-linked streptavidin was added to each well and incubated for 2 hours at room temperature. Membranes were then washed and then chemiluminescent detection reagents were added and exposed to film for 2–30 sec. Arrays were scanned into a computer and optical density measurements were obtained with National Institutes of Health ImageJ software.

4.9 Endothelial cell proliferation by CRC infiltrating NK derived conditioned media

10^3 umbilical vein endothelial cells (HUVECs) (Promocell, Heidelberg, Germany) were grown on 1% gelatin-coated 96 well tissue culture plates in M199 growth medium (Sigma-Aldrich, St Louis, MO, USA), supplemented with heat inactivated 10% FBS (Sigma-Aldrich), 2 mM glutamine (Gibco-Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), 100 μ g/ml heparin sodium salt (Sigma-Aldrich), 10 μ g/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml endothelial growth factor (EGF), 10 ng/ml acid and basic fibroblast growth factor (aFGF, bFGF) (Peprotech, Rocky Hill, NJ, USA). After cell adhesion, medium was replaced with 100 μ L of fresh medium, containing equal amount of NK CM and cultured up to 48 hours at 37°C, 5% CO₂. The 1-(4,5-

dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) reagent (Sigma Aldrich) was added to a final concentration of 0.5 mg/ml to each well. Following 3 hours incubation at 37° C, medium was removed and formazan crystals were dissolved with 100 µl of DMSO. Absorbance was read at 570 nm in a micro-plate reader SpectraMax M2 (Molecular Devices, Sunnyvale CA).

4.10 Chemotaxis and Morphogenesis on Human Umbilical Vein Endothelial Cell

Conditioned media (CM) were obtained from PB (either from healthy age-matched controls or CRC patients), normal adjacent and tumor tissues FACS-sorted NK cells, as above. We evaluated the ability of NK-derived CM to induce endothelial cells recruitment using the Boyden chamber assay. 5×10^4 human umbilical vein endothelial cells (HUVECs; Promocell, Heidelberg, Germany) were placed onto the upper compartment of a Boyden chamber apparatus. A 10-µm pore size polycarbonate filter, pre-coated with collagen I (50 µg/ml), was placed as an interface between the upper and lower chamber. CM obtained from HC (healthy controls), PB (peripheral blood of CRC patients), ADJ (adjacent tissue) and TUM (tumor tissue) sorted NK cells were used as a chemoattractant, while serum free RPMI 1640 and 10% FBS supplemented RPMI 1640 were used respectively as a negative and positive controls. Following 6 hours of incubation at 37°C and 5% CO₂,

the filters were recovered, and the cells migrated to the lower filter surface stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) finally counted in a double-blind manner in five consecutive fields per filter, with a fluorescence microscope (Zeiss, Oberkochen, Germany).

When plated on reconstituted basement membrane layer (matrigel), endothelial cells are able to induce the formation of a capillary network in the presence of a pro-angiogenic stimulus. We used NK CM to assess their ability to induce morphogenesis on matrigel (BD) layered human umbilical vein endothelial cells (HUVECs). 5×10^4 HUVE cells were seeded on a 10 mg/mL matrigel pre-coated 24 well plate and exposed on equal amount of NK CM obtained from HC, PB, adjacent and tumor tissue samples for 6 hours at 37°C and 5% CO₂. After six hours, the network formation was detected with an inverted microscope (Zeiss). Master segment numbers and length were quantified using ImageJ software and the angiogenesis analyser tool.

4.11 Cell adhesion assay *in vitro*

HUVECs (3×10^3 cells) were exposed to NK derived CM and seeded in a 48 wells plate pre-coated for 45 min with fibronectin (2 µg/ml). After 90 min incubation, the supernatants were removed and cells were washed with PBS. Cells were fixed with 4% paraformaldehyde and stained with DAPI. Assays were performed in triplicates wells. Fifteen microscope

fields were randomly selected from three wells for each treatment to count the number of adherent cells.

4.12 Flow Cytometry and Statistical Analyses

Flow cytometry analyses were performed using BD FACSDiva (v6.1.2) and FlowJo (v7.2.5) software. Statistical analyses were performed using the GraphPad Prism5 statistics and graphing program (GraphPad Software, San Diego, CA). Two-tailed t tests were used for comparison of paired data sets and quartile localization for population distribution or one-way ANOVA for multiple data sets, and quartile localization for population distribution. For the morphogenesis analysis, master segment number and length were quantified using the ImageJ software and the angiogenesis analyser tool. Data obtained with secretomic and pathscan immunoassay were quantified using the ImageJ software and the Dot Blot analyser tool.

Results

5. RESULTS

5.1 CD56⁺CD16⁻ NK cells represents the predominant subset within tumor tissues

Initial flow cytometric analysis of PBMC from HC and CRC patients was performed to evaluate NK cell subset distribution (Figure 10 A). We observed that NK cells represent 0.1% to 3% of the whole CD45⁺ leukocytes population within the tumors, 1% to 4% of adjacent tissue (ADJ), and 13% on average in the peripheral blood (Figure 10 B). An increased proportion of CD56⁺CD16⁻ NK cells were found in ADJ (87.9%) and in TUM (94.3%) samples as compared with healthy donors (16.3%) and PB of CRC patients (4.46%), while the CD56⁺CD16⁺ cytotoxic NKs remain the predominant subset in peripheral blood samples from patients and HC (Figure 10 B).

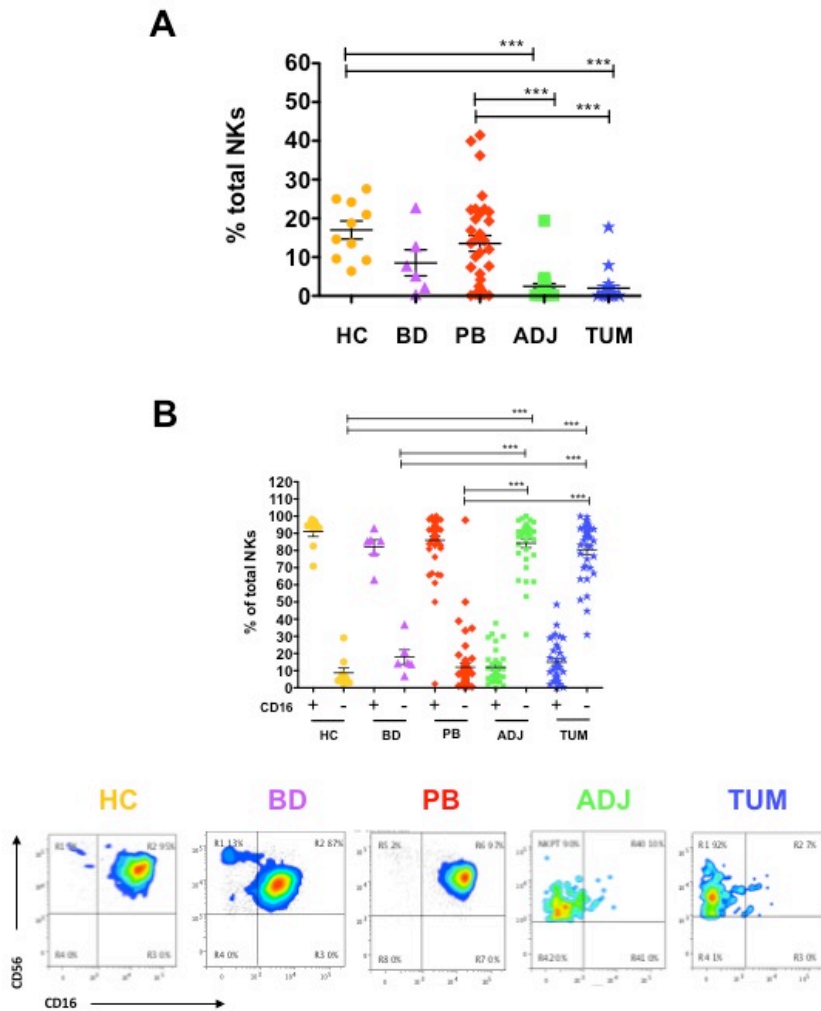


Figure 10- Subset analysis of NK cells in healthy donors and CRC derived samples. A) number of NKs in peripheral blood of healthy controls (HC), non-oncologic patients with other bowel diseases (BD), CRC patients (PB), adjacent tissues (ADJ) and tumor biopsies (TUM). **B)** percentage of CD56⁺CD16⁺ and CD56⁺CD16⁻ in HC, BD, PB, ADJ and TUM samples. Results are expressed as Mean±SEM. n= 4-50. Statistical analysis: one-way ANOVA ***=p<0.001

5.2 CRC-derived NK cells show an impairment of cytotoxicity compared with NK isolated from healthy controls

We therefore investigated whether the induction of TINK/TANK phenotype in CRC correlates with impaired NK function. We found that NKG2D, a surface marker for NK cell activation, was significantly decreased in NK cells isolated from peripheral blood, adjacent normal and tumor tissues from patients with CRC (* $P < 0.05$), as compared to healthy controls (Figure 11 A). NKG2D decreased expression on CRC NK cells correlates with impaired NK killing efficiency, as confirmed by the down-regulated CD107a expression levels (Figure 11 B), a surface marker for NK cell degranulation, on clinical samples.

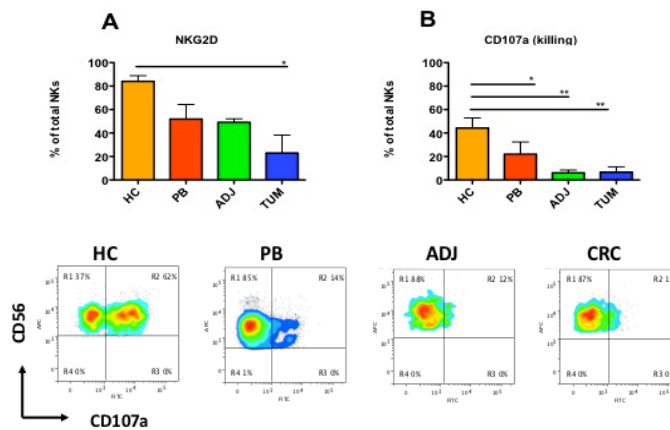


Figure 11 Evaluation of cytotoxic activities of NK cells in healthy donors and CRC samples. TINKs and TANKs isolated from CRC adjacent and tumor tissue samples show **A)** decreased level of the activatory NKG2D molecule that is associated **B)** with functional impairment of their killing activity measured by flow cytometry analysis of CD107a levels. HC: NK isolated from peripheral blood of healthy controls, PB: NK isolated from peripheral blood of CRC patients. ADJ: NK isolated from adjacent tissues, TUM NK isolated from tumor biopsies. Results are expressed as Mean \pm SEM. n= 3-6. Statistical analysis: one-way ANOVA *= $p < 0.05$; **= $p < 0.01$

5.3 NKs isolated from CRC tissues show a decidual-like phenotype

Given the similarities between CRC TINKs/TANKs with dNK, we evaluated whether NK from CRC samples were able to express the decidual NK markers CD9 and CD49a. We demonstrated that NK cells derived from CRC samples exhibit a decidual-like phenotype, showing increased expression for the dNK markers CD9 and CD49a, difference that is statistically significant when considering only CD56⁺CD16⁻ subset (*P<0.05; **P<0.01; ***P<0.001) (Figure 12).

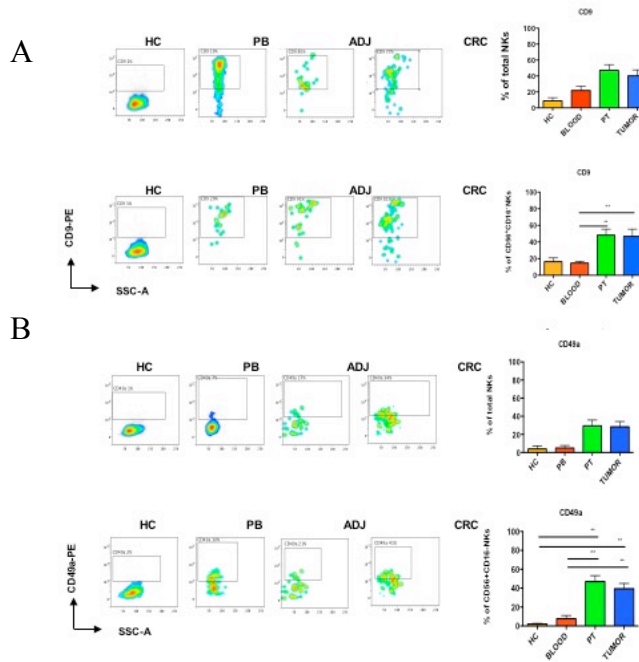


Figure 12 Decidual-like phenotype evaluation of CRC NK cells The CD56⁺CD16⁻ TINK and adjacent NK cells express higher levels of the CD9 (A) and CD49a (B) NK decidual markers as compared with those from peripheral blood NK cells (patients and healthy controls). HC: NK isolated from peripheral blood of healthy controls, PB: NK isolated from peripheral blood of CRC patients, ADJ: NK isolated from adjacent tissues, TUM NK isolated from tumor biopsies. Results are expressed as Mean±SEM. n=3-10. Statistical analysis: one-way ANOVA *p<0.05; **p<0.01, ***p<0.001.

Similar results are also found on TINKs and TANKs from NSCLC patients, thus supporting the hypothesis of a decidual-like subset in solid tumors (Figure 13).

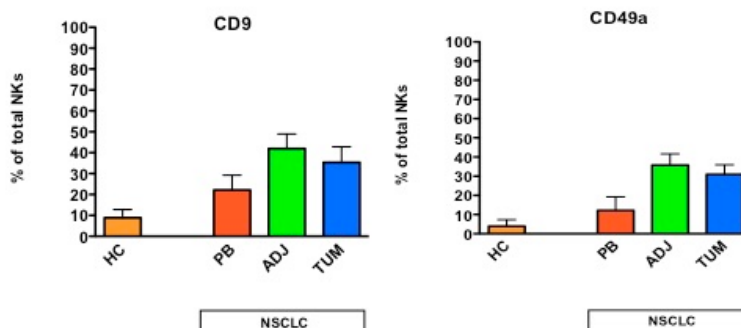


Figure 13 Decidual-like phenotype evaluation of non small cell lung cancer (NSCLC) NK cells. According to our results obtained in CRC derived NKs, the CD56⁺CD16⁻ TINK and adjacent NK cells express higher levels of the CD9 and CD49a decidual markers as compared with those from peripheral blood NK cells (patients and healthy controls). HC: NK isolated from peripheral blood of healthy controls, PB: NK isolated from peripheral blood of NSCLC patients, ADJ: NK isolated from adjacent tissues, TUM NK isolated from tumor biopsies. Results are expressed as Mean±SEM. n= 3-7. Statistical analysis: one-way ANOVA *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$.

5.4 CRC derived TANKs can release pro-angiogenic factors

To further evaluate the pro-angiogenic properties of CRC derived NKs, we performed secretomic analysis using conditioned media from CRC patients and HC FACS sorted NKs, using commercially available membrane array kits. Since the limited percentage of NK cells in CRC biopsies, for molecular studies, we focused our attention on CRC derived

TANKs, comparing them with NK cells isolated from healthy donors. Secretomic analysis revealed that CRC TANKs are able to release a wide array on pro-angiogenic factors, necessary for endothelial and inflammatory cell recruitment and proliferation (including GRO, MCP-1, ENA-78, EGF, I-309), as compared to HC. Among these, a strong up-regulation was observed for Angiogenin, IL-8, TIMP-1 and TIMP-2, MMP-9, MCP-1, VEGF and Angiopoietin 1 (Figure 14).

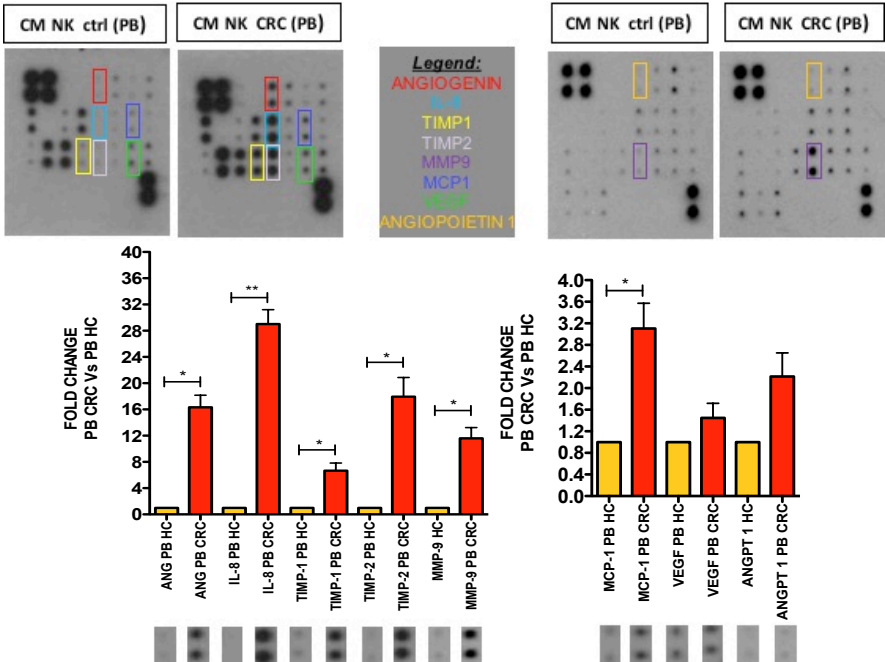


Figure 14 Secretome analysis by protein membrane arrays – peripheral blood NK cells isolated from CRC patients are able to release a wide array of pro-angiogenic factors as compared to healthy controls. Data obtained were quantified by ImageJ Dot Blot analysis software and factors that resulted highly up-regulated were selected to be validated. Orange bars: supernatants from healthy controls derived NKs, red bars: supernatants from peripheral blood NK cells isolated from CRC patients. Results are expressed as percentage over ctrl (HC). Statistical analysis: t-test *=p<0.05

Therefore, to validate these data we performed intracellular staining for cytokine detection using a multicolour flow cytometry approach. Our results confirm data obtained with secretomic analyses, showing a significant increase of selected cytokine and factor production by TANKs comparing them with NK isolated from healthy controls. To further investigate whether the production of pro-angiogenic factors by CRC derived TANKs may represent a relevant parameter to keep into consideration, we also included in our cohort non-oncologic patients with other bowel diseases (BD) (diverticulosis), showing that NK cells isolated from patients with BD patients produced comparable levels of pro-angiogenic factors with that of healthy controls. On the contrary, a significant enhanced production of Angiopoietin, IL-8, TIMP-1, TIMP-2, VEGF and MMP-9 was observed comparing CRC TANKs to BD NKs (Figure 15) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

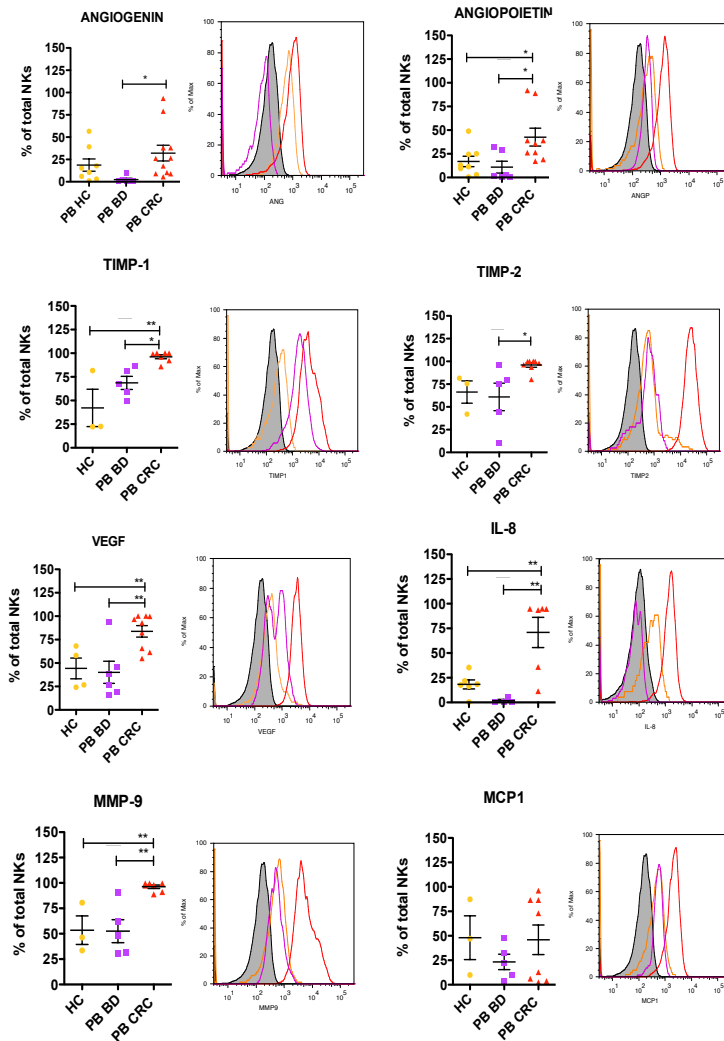


Figure 15 Pro-angiogenic cytokines production by NK cells- Pro-angiogenic cytokines production was assessed by multiparametric flow analysis, confirming that peripheral blood NK cells isolated from CRC patients (PB CRC) can produce higher levels of selected cytokines compared with peripheral blood NK cells isolated from healthy donors (HC). In addition, CRC TANKs showed an increased production of pro-angiogenic molecules also when compared to NK isolated from patients with non-oncologic bowel diseases (PB BD). Results are expressed as Mean±SEM. n= 3-11. Statistical analysis: one-way ANOVA *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$.

These data clearly demonstrate that a pro-angiogenic polarization occurs on peripheral blood CRC NKs, suggesting a systemic effect on NK cells from CRC patients. These data indicate a potential application of NK cells as potential clinical biomarkers of CRC.

5.5 CRC TINK/TANKs Promote Angiogenesis-Associated Effects on Endothelial Cells (HUVECs)

We further investigated whether CRC TINK/TANKs, showing a pro-angiogenic phenotype in term of surface antigen expression and cytokine profiling, were effectively able to induce angiogenesis *in-vitro*. We found that conditioned media (CM) obtained from FACS sorted NK from CRC peripheral blood and tumor tissues are able to induce HUVE cell proliferation as compared with those from healthy controls (Figure 16).

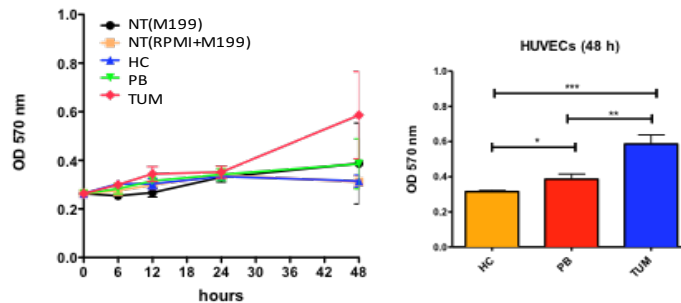


Figure 16 Pro-angiogenic activity of NK cell derived conditioned media – induction of HUVEC proliferation. Supernatants from CRC NK cells induced endothelial cell proliferation after 48 hours of exposition. HC, serum free M199 medium supplemented with 30% of healthy controls derived NK supernatants. PB, serum free M199 medium supplemented with 30% of supernatants of CRC TANKs. TUM, serum free M199 medium supplemented with 30% of supernatants of CRC TINKs. Results are expressed as Mean±SEM. Statistical analysis: t-test *= $p < 0.05$; **= $p < 0.01$

Migration is a crucial step in the angiogenic process, since endothelial cells are required to migrate from pre-existent vessels to the tumor site. CM from CRC patients NK cells, either from peripheral blood or tumor infiltrating, were able to recruit more endothelial cells as compared with those from HC, as confirmed by the Boyden Chamber migration assay (Figure 17).

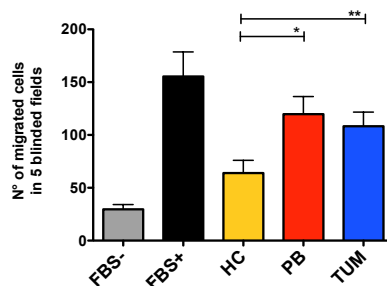


Figure 17 Pro-angiogenic activity of NK cell derived conditioned media – induction of HUVEC migration. Supernatants from CRC NK cells induced endothelial cell migration. FBS-, serum-free medium as a negative control. FBS+, serum-free M199 with 1% L-glutamine, fibroblast growth factors (10 ng of acidic fibroblast growth factor plus 10 ng of basic fibroblast growth factor/ml), epidermal growth factor (10 ng/ml), heparin (0.1 mg/ml), and hydrocortisone (0.1 µg/ml). HC, serum free M199 medium supplemented with 30% of healthy controls derived NK supernatants. PB, serum free M199 medium supplemented with 30% of supernatants of CRC TANKs. TUM, serum free M199 medium supplemented with 30% of supernatants of CRC TINKs. Results are expressed as Mean±SEM of two independent experiments. Statistical analysis: t-test *=p<0.05; **=p<0.01

When cultured on a membrane-basement matrix layer, HUVE cells can form capillary-like structures *in-vitro*, mimicking the events occurring during the vessel lumen formation *in-vivo*. We found that CM from NK isolated from CRC peripheral blood and tumor tissues are able to more efficiently induce the formation of capillary-like structures in HUVE cells, as compare with those from HC (Figure 18).

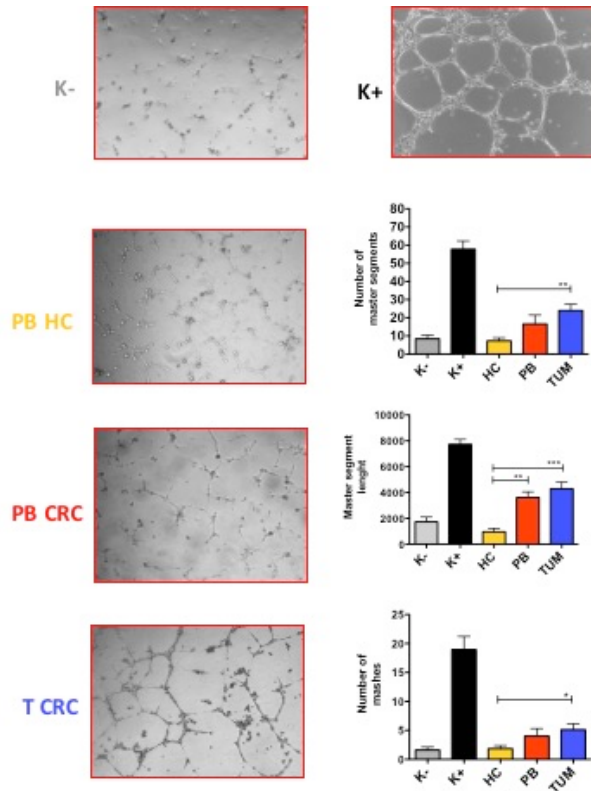


Figure 18 - Pro-angiogenic activity of NK cell derived conditioned media – induction of capillary-like structure formation by HUVECs. Supernatants from TINK/TANK promote morphogenesis process on HUVE cells plated on a matrigel basement. K-, serum-free medium as a negative control. K+, serum-free M199 with 1% L-glutamine, fibroblast growth factors (10 ng of acidic fibroblast growth factor plus 10 ng of basic fibroblast growth factor/ml), epidermal growth factor (10 ng/ml), heparin (0.1 mg/ml), and hydrocortisone (0.1 μg/ml). HC, serum free M199 medium supplemented with 30% of healthy controls derived NK supernatants. PB, serum free M199 medium supplemented with 30% of supernatants of CRC TANKs. TUM, serum free M199 medium supplemented with 30% of supernatants of CRC TINKs. Results are expressed as Mean±SEM of two independent experiments. Statistical analysis: One-way ANOVA *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$

Finally, we demonstrated that TINK/TANK CM can significantly increase HUVEC adhesion on a fibronectin matrix (Figure 19) comparing with HC-NK CM ($***P<0.001$).

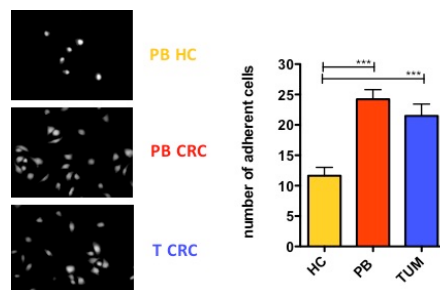


Figure 19 Pro-angiogenic activity of NK cell derived conditioned media – promotion of HUVEC adhesion. Supernatants from TINK/TANK promote adhesion of HUVE cells plated on a fibronectin matrix. HC, serum free M199 medium supplemented with 30% of healthy controls derived NK supernatants. PB, serum free M199 medium supplemented with 30% of supernatants of CRC TANKs. TUM, serum free M199 medium supplemented with 30% of supernatants of CRC TINKs. Results are expressed as Mean±SEM of three independent experiments. Statistical analysis: One-way ANOVA $***=p<0.001$

5.6 CRC TANK derived conditioned media are able to induce angiogenesis by activating diverse intracellular pathways in human endothelial cells

By using the PathScan Intracellular Signaling array kit to simultaneously detect the phosphorylation/activation of diverse pathways, we observed that conditioned media derived from FACS sorted TANKs are able to activate several pro-angiogenic pathways in HUVE cells. After 24 hours, phospho-(AMP-activated protein kinase α (AMPK α)), phospho-Glycogen Synthase Kinase 3 β and phospho-P70 S6 Kinase were up-regulated in

TANK CM exposed HUVECs compared with HC CM treated cells. Following 48 hours of exposition only phospho-AMPK and phosphor-S6 ribosomal proteins were up-regulated in HUVE cells treated with TANK CM (Figure 20).

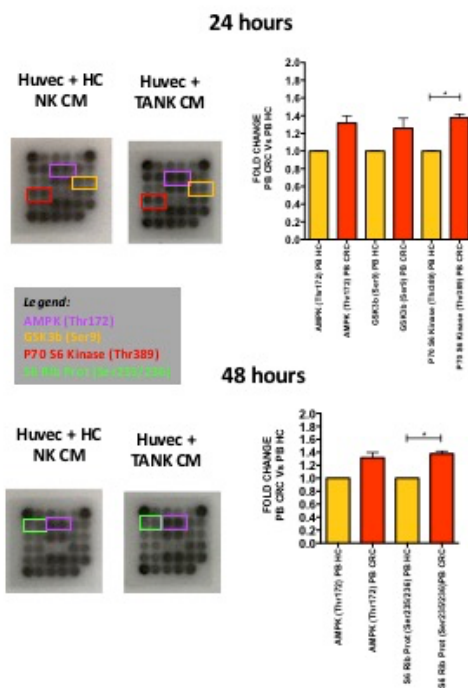


Figure 20 Intracellular Signaling activation in HUVEC treated with conditioned media derived by healthy controls and CRC TANK. Increased activation of several pathways, including AMPK, GSK3b, P70 S6 Kinase and S6 ribosomal protein was detected in HUVEC treated with CM from FACS sorted TANKs following 24hours and 48 hours of exposition. Data obtained were quantified by ImageJ Dot Blot analysis software. Orange bars: HUVECs exposed to CM derived from healthy control NK cells, red bars: HUVECs exposed to CM derived from TANKs. Results are expressed as percentage over ctrl (HC). Statistic analysis: t-student $*=p<0.05$

5.7 CRC TANKs showed diverse activated intracellular pathways compared with NKs isolated from healthy donors

We speculate that the pro-angiogenic phenotype of CRC TANKs and functions might be correlated with the activation of different molecular pathways able to regulate the transcription of pro-angiogenic molecules. To verify our hypothesis, we use the PathScan Intracellular Signaling array kit to simultaneously detect the phosphorylation/activation of diverse intracellular signaling.

Our data showed an increased phosphorylation/activation of several intracellular pathways in CRC TANKs compared with healthy control NK cells. Among these, several molecules involved in the regulation of NK metabolism (AMP-activated protein kinase α (AMPK α) and Pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1)) and cytotoxic functions (Phosphatase and tensin homolog (PTEN), Protein kinase B (AKT) and Protein Kinase B (mTOR)) (Figure 21) resulted more activated in CRC TANKs than in healthy control NKs.

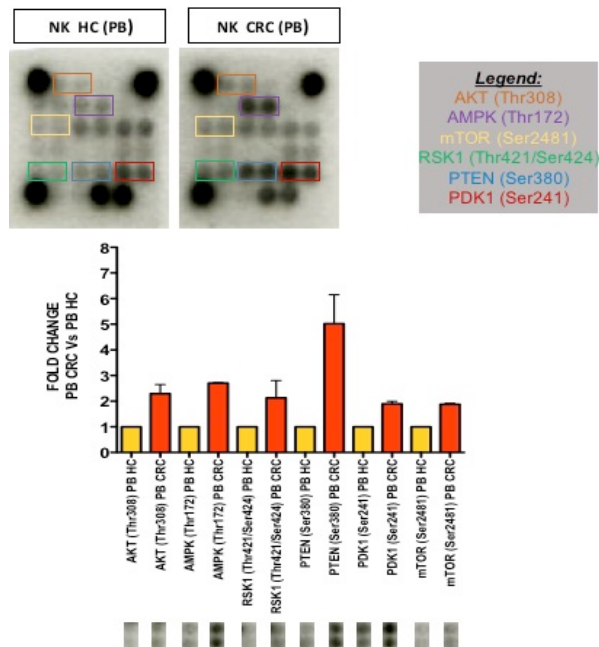


Figure 21 Intracellular Signaling activation in CRC TANKs. NKs isolated from CRC patients' peripheral blood showed an increased activation of several pathways, including AMP-activated protein kinase α (AMPK α) and Pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1), Phosphatase and tensin homolog (PTEN), Protein kinase B (AKT) and mammalian target of rapamycin (mTOR) Data obtained were quantified by ImageJ Dot Blot analysis software. Orange bars: NKs from healthy controls, red bars: NK cells isolated from peripheral blood of CRC patients. Results are expressed as percentage over healthy control (HC).

We further evaluated the expression and activation of STAT-3 and STAT-5, two molecules that have been reported to be related with NK cell ability to induce tumor angiogenesis and to sustain tumor progression [41, 42]. By using a multicolour flow cytometry approach, we observed a statistically significant increase of STAT-3 and STAT-5 expression and activation/phosphorylation in CRC TANKs compared not only with

healthy donors derived NK cells, but also with NK cells isolated from non-oncologic BD patients (Figure 22).

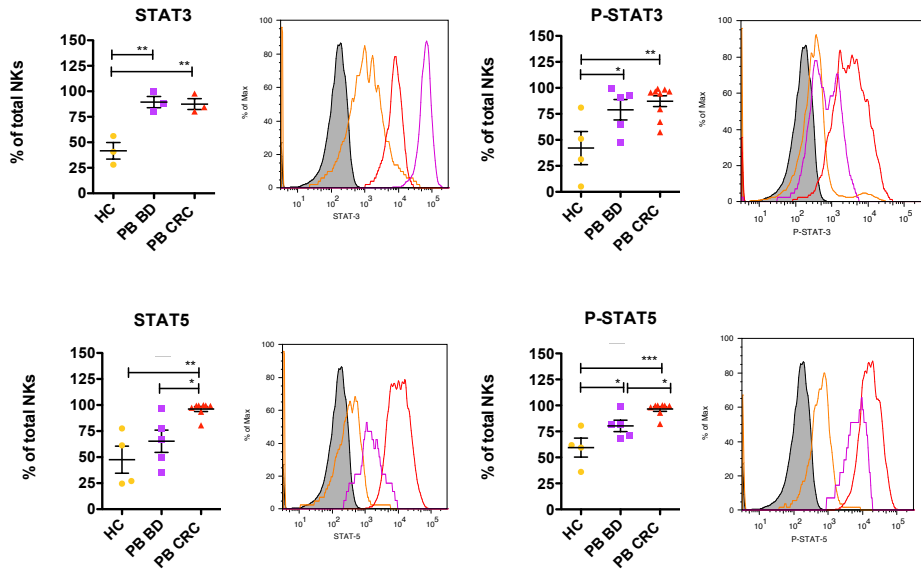


Figure 22 STAT-3 and STAT-5 expression levels and activation in NK cells- Peripheral blood NK cells isolated from CRC patients (PB CRC) showed higher levels of STAT-3 as far as STAT-5 compared with peripheral blood NK cells isolated from healthy donors (HC) and with NKs isolated from patients with non-oncologic bowel diseases (PB BD). Results are expressed as Mean \pm SEM. n= 3-11. Statistical analysis: One way ANOVA *= p <0.05; **= p <0.01, ***= p <0.001.

Discussion

6. DISCUSSION

Tumors represent an extremely heterogenic “scenario” where several “actors” interact each other inducing the complex network of tumor microenvironment (TME) [66, 75]. As a consequence of their plasticity, TME is able to shape immune cell functions that can acquire tumor-promoting features like anergy [76] and induction of angiogenesis [77]. The angiogenic switch has been described for diverse immune cells within the TME [76], while acquisition of angiogenic-features by NKs have been poorly investigated in the tumor context. The pro-angiogenic CD56^{bright}CD16⁻VEGF^{high}PIGF^{high}IL-8⁺IFN γ ^{low} NK cell subset was identified in patients with non small cell lung cancers [30, 33], placing NKs as a novel orchestrator in the inflammatory angiogenic switch in tumors. TGF β , a major immunosuppressive cytokine in the TME act and a pro-angiogenic switcher on cytolytic NK both at tissue and peripheral levels. Based on these novel findings on NSCLC, we moved to CRC to investigate whether induction of pro-angiogenic phenotype and functions on peripheral blood and tissue infiltrating NKs may occur in other solid cancers. We confirmed previous data on NK subset distribution in CRC [74] and using a larger sample size we demonstrated that in normal adjacent and CRC tissues, NK cells are polarized towards the TINK/TANK CD56⁺CD16⁻ phenotype.

NK cells represent 0.1% to 3% of the whole CD45⁺ leukocytes population within the tumors, 1% to 4% of adjacent tissues, and 13% on average in

the peripheral blood. Our results on the low percentage of TINKs in CRC are supported by previous studies that have investigated NK infiltration in colorectal tumors and the possible correlations with the clinical outcome. Contrasting results, correlating the number of infiltrating NKs and CRC patients outcome are present in the literature. Sandel et al. [28], and Halama et al. [72] observed a low percentage of NKs within CRC tissues that appears to exert a marginal role for NKs in tumor regulation [28, 62]. On the contrary, the relevance of NK on patient outcome were suggested by Gulubova et al. and Menon et al. which revealed that NKs, despite their low number, play a crucial role in the immune surveillance in colorectal cancer patients, showing that NK infiltration of tumor tissues is associated with a reduced risk of relapse and prolonged survival [78, 79]. Marechal et al., demonstrated that CD56⁺ cells and mainly NK cells are the major effector of ADCC related-cetuximab activity, suggesting that CD56⁺ cells infiltrate in primary colorectal adenocarcinoma may represent a relevant parameter to monitor to predict the response of mCRC patients treated with first-line cetuximab-based chemotherapy [80]. Sconocchia et al highlight the relevance of the cross talk between intra-tumor NK cell infiltration and other immune cells, including T lymphocytes within CRC. They show that NK cells positively influenced the clinical outcome of CRC patients by enhancing the protective role of tumor-infiltrating CD8⁺ T cell [62]. In agreement with this evidence, Van Den Broeke et al reported that NK/dendritic cell (DC) interaction

represent an interesting mechanism against cancer, suggesting in an *in vivo* model of colorectal cancer that mature DCs may stimulate CD4⁺ T cells that could in turn directly activate NK cells through IL-2 secretion [81]. Confirming the phenotype and function similarity between dNK and NSCLC/CRC TINK/TANKs, we found that in tumor and normal adjacent tissues, CRC NKs exhibit increased expression of the dNK markers CD9 and CD49a [36, 37, 82], suggesting that the polarization towards a TINK/TANK phenotype may represent a crucial feature of solid tumors. These data are supported by Levi et al., which recently reported that a clear and significant enrichment of the CD56^{bright}CD16^{dim} NK subset expressing decidual surface markers occurs in a small cohort of CRC, breast and melanoma patients [83]. Several studies have showed that NKG2D engagement is crucial for NK cell activation and that decreased NKG2D expression is associated with impaired NK cell function in gastric cancer [84], metastatic melanoma [85] and breast cancer [32], resulting in cancer immune-escape. We showed that NKG2D expression is decreased in peripheral blood, adjacent normal and tumor tissues of CRC patients as compared to healthy controls. This different pattern of NKG2D expression correlates with impaired cytotoxicity, as confirmed by the degranulation assay, showing decreased CD107a levels in all CRC samples. We suggest that the alteration in NKG2D receptor repertoire and impairment of NK lytic activity might reflect the polarization that induce NK to sustain tumor progression through angiogenesis induction. Our

data are supported by Gharagozloo et al and Rocca et al, which also showed a significant reduction in the percentage of NKG2D⁺NK cells as well as NKG2D mRNA expression in peripheral blood of metastatic colon cancer patients [74, 86], suggesting that the decreased expression of activating NKG2D receptor in CRC might represent a relevant marker in the clinic. NK cytotoxicity impairment in CRC TANKs was also confirmed by the alteration of intracellular pathways, including phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and AMP-activated protein kinase (AMPK) signaling, that have been reported to negatively regulate NK cytotoxicity. Briercheck et al showed up-regulation of PTEN in resting CD56^{bright} NK cells [87]. Leong et al suggested PTEN as a key regulator of NK killing functions by promoting IFN- γ production in response to cytokine stimulation and migration to distal tumor sites [88]. Likewise, AMPK activation has been associated with the reduction of NK cytotoxicity, cytokine secretion, proliferation, and telomerase expression [89]. Extensive alterations in serum cytokine repertoire have been reported in CRC patients, suggesting their direct contribution to cancer progression [90, 91]. These studies highlighted their implication in the clinic and their potential use as targets for the design of novel therapeutic approaches or as biomarkers able to strengthen the prognosis of the Immunoscore [92]. According with these findings, we observed an increase in several cytokines and chemokines release by CRC TANKs, including VEGF, IL-8, MMP-9, TIMP-1 and

TIMP-2, angiogenin and angiopoietin 1, that could contribute to angiogenic induction in CRC. The study by Asfaha et al supports this observation: they showed an up-regulation of IL-8 expression in CRC tissue compared with adjacent healthy colonic tissue, demonstrating that IL-8 can exacerbate inflammation and accelerates colon carcinogenesis by promoting tumor angiogenesis [93]. Likewise, VEGF up-regulation has been reported CRC tissue and positively correlates with advanced tumor stage as well as positive lymph node and liver metastasis [94]. VEGF plasma levels are higher in CRC patients compared with healthy donors and have been correlated with a negative outcome [95]. Secreted VEGF is also able to activate STAT-3 signaling in CRC tumors [96] promoting cancer progression and induces angiogenesis by acting directly or indirectly on endothelial cells [97]. Based on our results and literature data, we hypothesized a role for NK cells in CRC progression acting by angiogenesis induction, and we observed that FACS sorted NK derived conditioned media (CM) from CRC TINK/TANKs are able to promote angiogenesis *in vitro*, by inducing HUVEC proliferation after 48 hours of exposition. This phenomenon is associated with the increase of S6 ribosomal Protein levels that represents a positive regulator of endothelial cell proliferation [98]. TANK CM also induces the formation of capillary like structures and promotes endothelial cell migration and adhesion by inducing AMPK and GSK3 β phosphorylation. The correlation between NK and angiogenesis has been also demonstrated by previous work on

NSCLC, obtaining comparable results [30, 33]. The relevance of NK in tumor angiogenesis have also been highlighted by Gotthard et al, that clarified in an *in-vivo* model of RMA-S lymphoma cells and *v-abl* transformed tumor the crucial role of STAT-5 pathway [41, 42]. They demonstrated that *Stat5^{ΔΔ}Ncr1-iCre^{Tg}-Vav-Bcl2* mice failed to control tumor growth and produced increased VEGF levels [41, 42]. In accordance with these findings, we also investigated the percentage of total STAT-5 and P-STAT5 positive NK cells, hypothesizing the same mechanism of action. In contrast to what we expected, we found increased levels of STAT-5 in TANK compared with NK from healthy donors. Also P-STAT3 levels, another key regulator of NK development, activation and target cell killing, were up-regulated in tumor derived NK compared with healthy controls. Persistent activation of STAT-3 has been reported to enhance tumor cell proliferation, survival and invasion while suppressing anti-tumor immunity by inducing NF-κB phosphorylation and up-regulating several molecules including IL-8, MMP-9, VEGF and MCP-1 [99]. The induction of STAT associated pathways has been reported in several tumors and among its activators, cytokines and Tyrosine Kinase Receptors signaling have been reported to play a crucial role [100]. Küçük et al also showed that activation of STAT3 and STAT5B due to activatory mutations occurs frequently in NK/T-cell lymphomas [101].

conclusions

7. CONCLUSIONS

Our study sheds light on the role of NKs in tumor progression, that a part from immunosuppression, includes induction of angiogenesis. Our results clearly demonstrate that pro-angiogenic phenotype and functions of TINK/TANK might represent a common feature of solid tumors that not only contribute, but also orchestrate other TME components to support tumor progression by enhancing tumor angiogenesis. In this view, NK cells and their associated cytokines might be considered for future studies aimed at investigation their potential application as prognostic parameter as well as clinical biomarkers, to early detect tumors or for patients' follow up. Therefore, the identification of NKs in the blood as “predictors” of those infiltrating solid cancers, allow the extension of the recent concept of immunoscore to the peripheral level suggesting the possibility of an inflammoscore. Finally, CRC NK cells may represent a potential therapeutic target for agents able to restore the cytotoxic phenotype/function of TINK/TANKs or interfere with their altered polarization state.

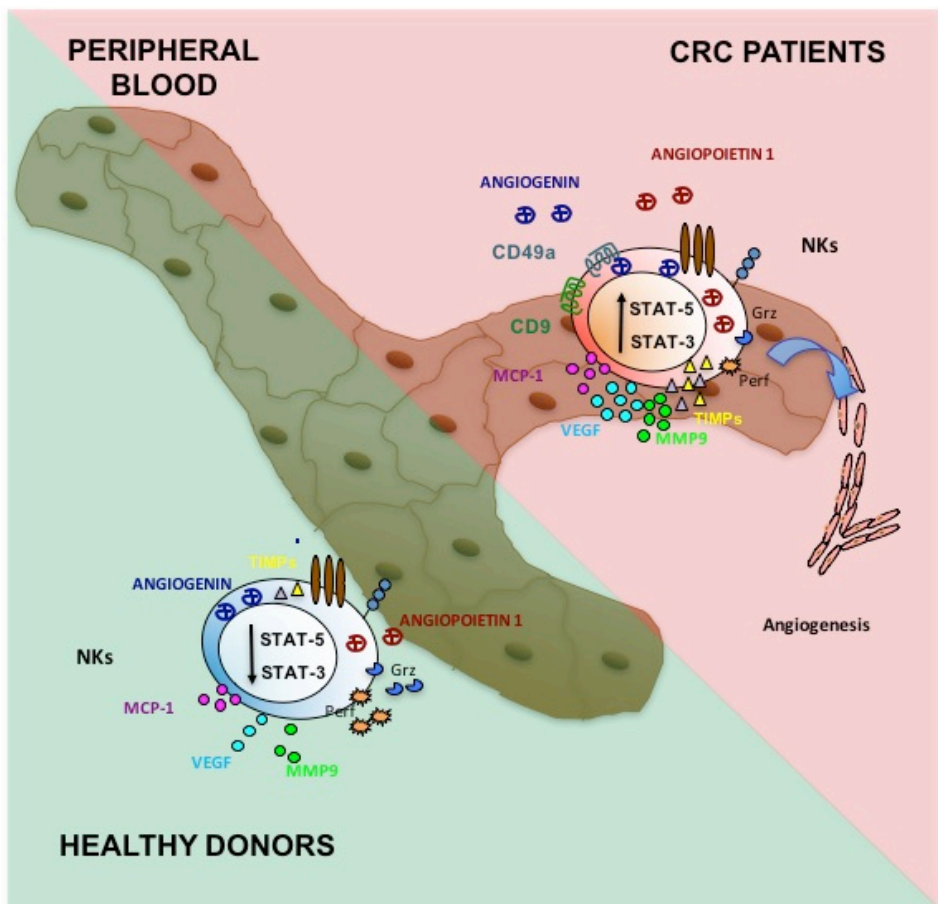


Figure 23 – Representative scheme of our findings

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